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Familial Hypercholesterolaemia: molecular and functional study of *LDLR* mutations

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ABSTRACT

Cardiovascular disease (CVD) remains the most common cause of death globally. Dyslipidaemia is one of the most important risk factors that leads to CVD. It can be due to a monogenic condition or to polygenic/environmental causes as diabetes, obesity, tobacco use, excess of alcohol or reduced physical activity. The identification of the individuals at risk and the distinction of these two types of dyslipidaemia is important for a correct cardiovascular risk assessment, counselling, and treatment reducing, this way, cardiovascular mortality.

Familial hypercholesterolaemia (FH) is an autosomal dominant disorder of cholesterol metabolism. Most commonly, FH results from inherited defects in the Low-Density Lipoprotein Receptor Gene (*LDLR*) leading to increased levels of circulating LDL cholesterol and lipid accumulation in arteries and tendons. Mutations in other genes as the apolipoprotein B gene (*APOB*) and proprotein convertase subtilisin/kexin type 9 gene (*PCSK9*), are also responsible for FH. The distribution pattern of apolipoprotein E gene (*APOE)* polymorphisms affects the affinity to lipoprotein receptors and, consequently, the clearance of dietary fat from the blood, also causing dyslipidaemia.

The homozygous form of FH is rare and more severe, but the heterozygous form is common, with a frequency of 1/500 in most of European countries, although underdiagnosed in several populations, including the portuguese.

FH is characterized by increased levels of plasmatic cholesterol since birth, which results in cholesterol deposits in extravascular tissues that can be identified in young patients (below 45 years old): xanthelasma, corneal arcus deposits and tendon xanthomas. This accumulation can cause premature arteriosclerosis and coronary heart disease (CHD). The presence of tendon xanthomas allows the differentiation of FH from other causes of hypercholesterolaemia as polygenic hypercholesterolaemia.

More than 1700 different alterations in *LDLR* gene have been described worldwide. However, the functional studies for the great majority of these variants, have not been performed. For patients carrying these variants, a definitive molecular diagnosis for FH is not possible, representing a serious problem for FH diagnosis.

In 1999, the Portuguese FH study was established at the National Institute of Health to identify the genetic cause of hypercholesterolaemia in individuals with a clinical diagnosis of FH. Index patients are included in this study using an adaptation of the Simon Broome (SB) criteria. Nonetheless, FH remains underdiagnosed and undertreated in the portuguese population.

The main aim of this project was to perform the molecular identification of genetic variants in *LDLR, APOB* and *APOE* genes, causing dyslipidaemia in patients referred to the Portuguese FH Study in 2015/2016 with a clinical diagnosis of FH, in order to improve the identification of individuals at risk. Functional studies in RNA for putative splicing variants were also performed.

The molecular diagnosis was performed for 60 index cases. Genomic DNA was isolated from peripheral blood lymphocytes using the salting out method. The 18 exons and promotor region of *LDLR*, part of exons 26 and 29 of *APOB* and exon 4 of *APOE* were amplified by PCR and sequenced by direct Sanger sequencing.

A total of 18 variants were identified in 24 of these patients. The cascade screening in relatives of these 24 index patients allowed the identification and genetic characterization of additional 19 FH patients in Portugal. All alterations found have been previously reported, although only 11 had been functionally assessed. The search for large rearrangements was performed by Multiplex Ligation-dependent Probe Amplification (MLPA). *In silico* analysis was performed for all the variants found.

In order to access the effect of splicing mutations, RNA was isolated from patients' blood with RNeasy® Mini Kit (Qiagen), after isolation of peripheral blood mononuclear cells, and then transcribed to cDNA. Regions of interest were amplified with specific primers designed to evaluate the effect on cDNA of two of the tree putative splicing variants found in *LDLR* gene. Specific detection of each transcript was accessed by an agarose gel and the fragments were sequenced by Sanger sequencing. Both alterations lead to skipping of an entire exon and create premature stop codons: c.1060+1G>A causes an inactivation of the donor site in intron 7 resulting in skipping of exon 7; the alteration in the last nucleotide of exon 16 (c.2389G>A) creates a new acceptor site causing the skipping of exon 16.

The early genetic identification of a mutation, confirming the clinical diagnosis of FH, is very important, especially for young patients, since they can receive appropriate dietary and lifestyle advice and adequate therapeutic measures providing them longer and better lives.

Keywords: Familial Hypercholesterolaemia, *LDLR*, molecular diagnosis, splicing mutations

RESUMO

As doenças cardiovasculares (DCV) afetam o funcionamento normal do coração e dos vasos sanguíneos. Existem vários tipos de doenças cardiovasculares, sendo as principais a doença das artérias coronárias e a doença das artérias do cérebro. A doença coronária é, maioritariamente, provocada por aterosclerose, uma doença progressiva e inflamatória que resulta na formação de placas ateroscleróticas. Agentes infeciosos ou concentrações elevadas de lipoproteínas de baixa densidade (LDL) no sangue podem levar ao aparecimento destas placas na parede interna das artérias impedindo a circulação sanguínea.

As doenças cardiovasculares são uma das causas de morte mais comum no planeta. Fatores de risco como o uso de tabaco, obesidade, prática de exercício físico reduzida, consumo excessivo de álcool, diabetes, hipertensão, stress e dislipidémia aumentam a probabilidade de ocorrência prematura deste tipo de doenças.

A dislipidémia é um dos mais importantes fatores de risco da aterosclerose, uma vez que se caracteriza por anomalias quantitativas ou qualitativas dos lípidos no sangue. A identificação de indivíduos em risco e o conhecimento da causa de hipercolesterolemia é de extrema importância para que estes indivíduos possam ser corretamente tratados evitando-se, assim, a morte devida a esta causa.

A Hipercolesterolemia Familiar (FH) é uma doença autossómica dominante do metabolismo do colesterol. A FH é hereditária e resulta, maioritariamente, de mutações no gene do recetor das lipoproteínas de baixa densidade (*LDLR*) cuja função é a remoção do colesterol LDL do plasma, transportando-o para o fígado, onde é processado. Ficando esta função afetada, os níveis de colesterol LDL circulante, aumentam. Mutações noutros genes como o gene da apolipoproteína B-100 (*APOB*) e da pro-proteína convertase subtilisina/quexina tipo 9 (*PCSK9*) são também causa, embora menos frequente, desta doença. Alterações no gene da apolipoproteína E (*APOE)* afetam a afinidade com os recetores de lipoproteínas e, consequentemente, a remoção do colesterol do sangue, podendo também causar dislipidémia.

A forma homozigótica da FH é rara e mais severa, mas a heterozigótica é comum embora sub-diagnosticada em muitas populações, nomeadamente na portuguesa. Estima-se que, na maioria dos países europeus, a prevalência destas duas formas seja 1/1000000 e 1/500 indivíduos, respetivamente. O colesterol total, na forma heterozigótica, varia entre 290 e 500 mg/dL (com LDL>190 mg/dl) e na forma homozigótica, habitualmente, encontra-se entre os 600 mg/dL e os 1000 mg/dL.

O nível elevado de colesterol no plasma resulta, frequentemente, na formação de depósitos de colesterol nos tecidos extravasculares que, por vezes, podem ser facilmente identificados em indivíduos ainda jovens (abaixo dos 45 anos): xantelasmas, arco corneano e, mais difíceis de reconhecer mas mais específicos, os xantomas nos tendões. A presença de valores altos de colesterol LDL desde o nascimento, característico desta doença, leva a um incremento do risco de doença coronária prematura. Este fenótipo permite diferenciar a FH de outras causas de hipercolesterolemia, nomeadamente da hipercolesterolemia comum ou poligénica, embora nem sempre seja fácil essa diferenciação.

Foram descritas, em todo o mundo, mais de 1700 mutações diferentes no gene *LDLR.* Contudo, grande parte destas não possuem estudos funcionais, o que impede o diagnóstico definitivo destes doentes.

Em 1999 iniciou-se no Instituto Nacional de Saúde Dr. Ricardo Jorge o Estudo Português de Hipercolesterolemia Familiar. Este estudo tem como objetivos a pesquisa de alterações genéticas que possam confirmar o diagnóstico clínico de FH em indivíduos da população portuguesa e a determinação da prevalência e distribuição da FH em Portugal. Os casos *índex* são incluídos no estudo caso cumpram os critérios adaptados de Simon Broome. Estes critérios categorizam a FH como "definitiva" ou "possível", sendo que a primeira se define, em adultos, por valores de colesterol total acima de 290 mg/dL ou de colesterol LDL acima de 190 mg/dL, e em crianças, até aos 16 anos de idade, por uma

concentração de colesterol total cima de 260 mg/dL ou de colesterol LDL acima de 155 mg/dL, com presença de xantomas no doente ou num familiar de primeiro ou segundo grau ou ainda quando existe evidência genética de uma mutação num dos 3 genes que cause FH. O diagnóstico "possível" requer a presença de níveis de colesterol acima destes valores, valores totais de colesterol acima dos 290 mg/dL num familiar de primeiro ou segundo grau e história familiar ou enfarte do miocárdio antes dos 50 anos num familiar de segundo grau ou antes dos 60 anos num familiar de primeiro grau.

Sempre que possível, após a identificação da possível alteração causadora de doença é feito o estudo funcional para as variantes de patogenicidade desconhecida, para que o diagnóstico seja o mais completo e definitivo possível, contribuindo para uma abordagem terapêutica mais personalizada.

O principal objetivo deste estudo foi a identificação molecular de variantes genéticas nos genes *LDLR, APOB* e *APOE* que provoquem dislipidémia. Os doentes referenciados para o Estudo Português de Hipercolesterolemia Familiar em 2015/2016 foram o alvo deste projeto. A realização de estudos funcionais ao nível do RNA para variantes que afetam o splicing foi também objetivo deste estudo.

Neste projeto, foram estudados 60 casos índex incluídos no Estudo Português de Hipercolesterolemia Familiar. O estudo molecular foi dividido em várias fases: 1. O DNA genómico é isolado a partir dos linfócitos do sangue periférico; 2. Os 18 exões, regiões adjacentes e o promotor do gene *LDLR*, parte dos exões 26 e 29 do gene *APOB* e o exão 4 do gene *APOE* foram amplificados por PCR e sequenciados pelo método de sequenciação direta de Sanger. As sequências foram analisadas em computador e comparadas com as sequências de referência de forma a detetar variantes que possam ser a causa desta doença, confirmando o diagnóstico clínico; 3. Estudo de grandes rearranjos por Multiplex Ligation-dependent Probe Amplification (MLPA).

Foram realizadas predições *in silico* para todas as alterações encontradas para prever o seu impacto ao nível da proteína. Para as alterações em regiões codificantes foram utilizadas as ferramentas PolyPhen-2, SIFT, PROVEAN e Mutationtaster. As ferramentas HSF, NNSSP e FSPLICE foram usadas para prever o efeito no *splicing* causado pelas alterações em regiões intrónicas.

Quando nenhuma mutação é encontrada nestes três genes e o doente apresenta um fenótipo agressivo, procede-se à pesquisa no gene *PCSK9* e em todo o gene *APOB*, representando a quarta fase do estudo molecular. Quando são encontradas variantes de patogenicidade desconhecida, são realizados estudos funcionais *in vitro* (fase 5). Embora o estudo completo dos genes *PCSK9* e *APOB* não tenha sido realizado durante este trabalho, foram feitos estudos funcionais ao nível do *RNA*.

No total, foram identificadas 18 variantes diferentes em 24 destes doentes: 16 no gene *LDLR*, 1 no gene *APOB* e 1 no gene *APOE*. Apenas 11 destas apresentam estudo funcional. Sempre que existia amostra disponível, foi feito também o estudo genético dos familiares, o que permitiu a identificação e caracterização genética adicional de 28 indivíduos, num total de 43 doentes com uma alteração possivelmente patogénica. Entre estas mutações encontram-se 3 *nonsense*, 12 *missense* e 3 que possivelmente afetam o *splicing*. Uma destas mutações foi descrita, pela primeira vez, em Portugal neste projeto. A análise de grandes rearranjos não revelou alterações deste tipo no grupo em estudo.

A confirmação dos efeitos causados ao nível do *splicing* foi feita para duas das três alterações de *splicing* encontradas no gene *LDLR* durante este projeto. Para tal, recorreu-se ao isolamento das células mononucleares do sangue periférico dos doentes e à extração do *RNA* utilizando o RNeasy® Mini Kit (Qiagen). Após retrotranscrição para *cDNA* e amplificação da zona a estudar utilizando *primers* específicos, as bandas em gel de agarose foram analisadas e os fragmentos foram sequenciados. As duas alterações em causa levam ao *skipping* de exões e ao aparecimento de codões *stop* prematuros: no caso da alteração c.1060+1G>A, a inativação do donor site no intrão 7 resulta no *skipping* do exão 7; a alteração no último nucleótido do exão 16 (c.2389G>A) leva ao aparecimento de um novo *acceptor site* e, consequentemente, ao *skipping* do exão 16.

A FH é caracterizada por níveis elevados de colesterol plasmático desde a nascença. Por isso, é de extrema importância que o diagnóstico seja feito o mais cedo possível, principalmente em idade

pediátrica para que estas crianças recebam acompanhamento médico personalizado durante toda a vida, prevenindo o aparecimento de DCV prematura, permitindo, assim, uma melhor e maior esperança de vida. No entanto, é necessária uma maior divulgação da doença, principalmente junto do corpo clínico dos hospitais e centros de saúde assim como junto do público em geral. Só após a identificação clínica se pode realizar o estudo genético para comprovar a doença e o doente pode então receber acompanhamento e tratamento personalizado.

Palavras-chave: Hipercolesterolemia familiar, *LDLR*, diagnóstico molecular, mutações de *splicing*

ABBREVIATIONS

aa	Aminacid
ACAT2	Acyl-coenzyme A type 2
ACMG	American College of Medical Genetics
ApoAI	Apolipoprotein A I
ApoB	Apolipoprotein B protein
APOB	Apolipoprotein B gene
ApoCII	Apolipoprotein C II
ApoE	Apolipoprotein E protein
APOE	Apolipoprotein E gene
ARH	Autosomal Recessive Hypercholesterolaemia
bp	Base Pair
CABG	Coronary Artery Bypass Grafting
cDNA	Complementar Deoxyribonucleic Acid
CHD	Coronary Heart Disease
CVA	Cerebrovascular Accident
CVD	Cardiovascular Disease
Dab2	Disabled Homolog 2
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
EPFH	Estudo Português de Hipercolesterolaemia Familiar
ER	Endoplasmic Reticulum
ESE	Exon Splice Enhancer
FACS	Fluorescence Activated Cell Sorter
FH	Familial Hypercholesterolaemia
g	Gram
G	Relative Centrifugal Force
HC	Hypertrophic Cardiomyopathy
HDL	High-Density Lipoprotein
HDL-C	High-Density Lipoprotein Cholesterol
heFH	Heterozygous form of Familial Hypercholesterolaemia
HMG-CoA	3-Hydroxy-3-Methylglutaryl Coenzyme A
hoFH	Homozygous form of Familial Hypercholesterolaemia
HSF	Human Splicing Finder
IDL	Intermediate Density Lipoprotein
INSA	Instituto Nacional de Saúde Dr. Ricardo Jorge
kb	Kilobases
LCAT	Lecithin Cholesterol Acyltransferase
LDL	Low-Density Lipoprotein
LDL-C	Low-Density Lipoprotein Cholesterol
LDLR	Low-Density Lipoprotein Receptor protein
LDLR	Low-Density Lipoprotein Receptor gene
LDLRAP1	Low-Density Lipoprotein Receptor Adaptor Protein 1
Lp(a)	Lipoprotein (a)
LPL	Lipoprotein Lipase

ME	Myocardial Infarction
MEDPED	Make Early Diagnosis to Prevent Early Death
Min	Minutes
Mg	Miligram (10-6 g)
MLPA	Multiplex Ligation-Dependent Probe Amplification
mM	Milimolar (10-3 M)
mRNA	Messenger Ribonucleic Acid
NEC	Non Enzyme Control
ng	Nanogram (10-9 g)
NNSSP	Nearest-neighbor Secondary Structure Prediction
NTC	Non Template Control
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCSK9	Proprotein Convertase Subtilisin/Kexin Type 9
PolyPhen-2	Polymorphism Phenotyping 2
PROVEAN	Protein Variation Effect Analyser
PTCA	Percutaneous Transluminal Coronary Angioplasty
RNA	Ribonucleic Acid
RT	Room Temperature
SAP	Shrimp Alkaline Phosphatase
SB	Simon Broome
SDS	Sodium Dodecyl Sulfate
Sec	Seconds
SIFT	Sorting Intolerant From Tolerant
SNP	Single Nucleotide Polymorphism
TIA	Transient Ischemic Attack
TBE	Tris-Borate-EDTA
TG	Triglycerides
VLDL	Very Low-Density Lipoprotein
VUS	Variant of Unknown Significance
WHO	World Health Organization
W/V	Weight/Volume
ºC	Celsius Degree
µg	Microgram (10-6 g)
µL	Microliter (10-6 L)

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1 INTRODUCTION

1.1 Coronary Heart Disease and Atherosclerosis

Cardiovascular disease (CVD) affects the normal function of the heart and blood vessels. Ischemic heart disease (myocardial infarction and angina), cerebrovascular disease and heart failure are the principal categories. [1] Most cardiovascular diseases can be prevented by addressing social determinants of CVD such as tobacco use, unhealthy diet and obesity, physical inactivity and harmful use of alcohol. Other risk factors as hypertension, diabetes, stress and dyslipidaemia can also increase the probability of having premature CVD. [2]

Atherosclerosis is a slowly progressive disease of arteries that begins in childhood and does not manifest until middle-age or later. [1] It is an inflammatory disease, resulting from infection agents, smoking or high plasma concentration of lipoproteins as low-density lipoproteins (LDL), forming deposits in the inner wall of the arteries called atherosclerotic plaques. [3]

CVD remains the most common cause of death globally. [4] Since, in most cases, coronary heart disease (CHD) and atherosclerosis due to hypercholesterolaemia can be prevented, it is important to identify the individuals at risk and discover the cause of hypercholesterolaemia so they can be properly treated decreasing, this way, the CV mortality.

1.2 Lipoproteins and Cholesterol

A lipoprotein is a biochemical assembly that contains both proteins and lipids which carry the cholesterol in the bloodstream.

Cholesterol is an essential constituent in eukaryotic cell membranes, where it modulates fluidity and maintains the barrier between cell and environment. Furthermore, it is used for the manufacture of steroid hormones, vitamin D2 and bile acids. [2] It can be derived either from the intestinal absorption of dietary cholesterol, through the intestinal epithelial cells, or from synthesis *de novo* within the body. [5] In animal cells, cholesterol is biosynthesized through the mevalonate pathway that generally takes place in the endoplasmic reticulum (ER) of hepatic cells. [6] The enzyme Hydroxymethylglutaryl-CoA (HMG-CoA) reductase catalyses the limiting step in cholesterol synthesis. Hence, it has been a target for cholesterol-lowering drugs. [7]

Due to cholesterol insolubility in water, it cannot be readily mobilized when it gets accumulated, for example, within the wall of an artery, which can lead to the formation of atherosclerotic plaques leading to myocardial infarction and strokes. [2] Multicellular organisms solved the problem of cholesterol transport, both dietary and synthesized, by converting it into cholesteryl esters and packing it in the hydrophobic cores of plasma lipoproteins. This conversion is predominantly catalysed by the lecithin cholesterol acyltransferase (LCAT) in the peripheral tissues. [8] In the intestinal lumen, dietary cholesterol absorbed by enterocytes is esterified by acyl-coenzyme A: cholesterol acyltransferase type 2 (ACAT2). [9]

1.2.1 The Lipoprotein Pathways

The pathways of lipoprotein metabolism can be divided into four.

1.2.1.1 The Exogenous Pathway

In the dietary fat transport by the exogenous pathway, dietary triglycerides and cholesterol are processed in the intestinal lumen, absorbed, and packaged in the intestinal epithelial cell as nascent chylomicrons (90% of triglycerides and 3% of cholesterol). [10, 11] After exocytosis to the intestinal lymph, these lipoproteins acquire apolipoprotein E (apoE) from circulating high-density lipoproteins (HDL) and apolipoprotein C II (apoCII) which is a required co-factor of the enzyme Lipoprotein Lipase (LPL). [10] During lipolysis, the newly formed cholesteryl-ester enriched chylomicron remnants, lose 80-90% of triglyceride content and are removed from the bloodstream by receptors that recognize apoE. [12] After endocytosis, the cholesterol and triglycerides are hydrolysed in lysosomes, and the released cholesterol can be used to form bile acids, esterified and stored in the cell or used to downregulate HMG-CoA reductase. [13]

1.2.1.2 The Endogenous Pathway

By contrast, the endogenous pathway, which is responsible for the majority of cholesterol in circulation, requires *de novo* synthesis of cholesterol by the liver. [14] This results in secretion of nascent very low-density lipoproteins (VLDL) composed of triglycerides (70%), cholesterol (10%) and apolipoprotein B (apoB). [15] Like chylomicrons, circulating VLDL is hydrolysed by LPL resulting in VLDL remnants named IDL (intermediate density lipoprotein) particles that release free fatty acids. IDL particles can be cleared from the plasma by the hepatic LDL receptors (LDLR) that bind apoE. [16] The LDL formation (10% of triglycerides and 26% of cholesterol) occurs in the circulation from lipolysis of IDL and is accompanied by further loss of triglycerides, phospholipids and by the loss of apoE. Only apoB-100 is retained and constitutes the ligand for the LDLR. [14]

1.2.1.3 The LDL Receptor Pathway

LDL is the most abundant cholesterol-carrying lipoprotein in human plasma, being responsible for its delivery to all tissues. [17] Each LDL particle has a highly hydrophobic core consisting of polyunsaturated fatty acid known as linoleate and molecules of cholesteryl ester. This core is surrounded by a polar phospholipid coat and a single large protein: apoB. [18, 19]

LDL receptors are located on the cell surface, mainly in the liver, and specifically bind to apoB on the surface of the LDL (see Figure 1.1). [2, 20] The resulting receptor–ligand complex is then internalized by endocytosis forming a coated endocytic vesicle. [21] The endocytosis of LDLR is facilitated by LDL Receptor Adaptor Protein 1 (LDLRAP1). Due to the acid pH created by ATP-driven proton pumps, the LDLR separates from the LDL. [22] The receptor is recycled to the cell surface, whereas the LDL particle is degraded in the lysosomal compartment. The cholesterol esters present in the LDL particles are hydrolysed, the cholesterol is released and can be used for the numerous cellular functions listed before or stored in the form of cytoplasmic cholesteryl ester droplets until further use by the cell. Each cycle takes 10 minutes and LDLR has a 20-hour lifespan. [2]

LDLR also has affinity to proprotein convertase subtilisin/kexin type 9 (PCSK9) [23], secreted from hepatocytes. When present in the endocytic vesicle, binds the LDLR–LDL complex extracellularly and prevents it from dissociating, thus targeting the whole complex for degradation in the lysosomal compartment acting as a modulator of the LDLR pathway. [21, 24]

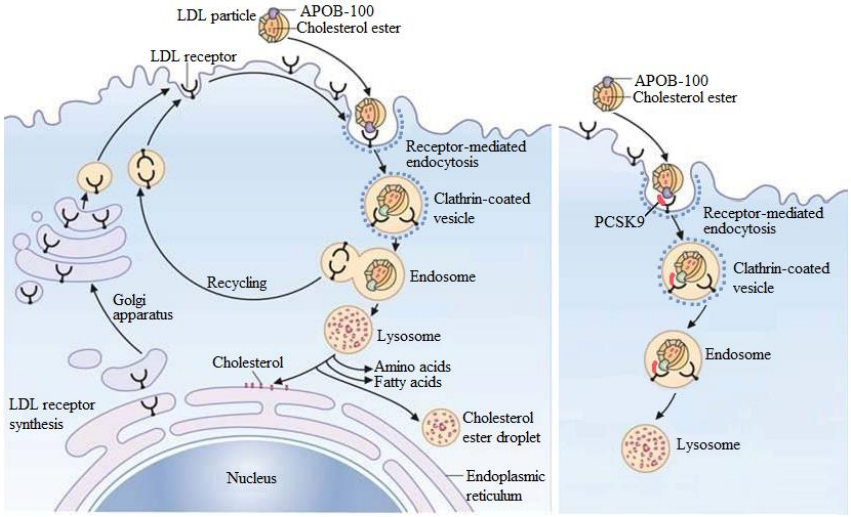

Figure 1.1 The LDL receptor pathway. On the left: LDLR synthesis occurs in the ER, undergoes extensive glycosylation in the Golgi apparatus and it is transported to the cell surface, where specifically binds to apoB in LDL particles. LDL is internalized by receptor-mediated endocytosis and the complex dissociates inside the endosome. The receptor is recycled to the cell surface and the LDL particle is degraded into the lysosome. On the right: PCSK9 is synthesized and excreted, binds to LDLR–LDL complex extracellularly, preventing the complex dissociation, leading to its degradation in the endosomal compartment. Adapted from [25]

1.2.1.4 The HDL Reverse Cholesterol Transport Pathway

This pathway provides a way for cholesterol, which cannot be metabolized by peripheral tissues, to move from these tissues back to the liver for excretion, maintaining the cholesterol homeostasis. [15] After the uptake of cellular cholesterol by the high-density lipoproteins (HDL), the cholesterol is esterified by LCAT. The liver captures the HDL cholesterol esters being here metabolized and excreted in the bile. [26]

1.3 Familial Hypercholesterolaemia

Genetic defects in genes codifying proteins involved in these lipoprotein pathways and the interaction between these variants with environmental factors, can lead to lipid disorders. Different inherited diseases of lipoprotein metabolism have been described and, for some of them, it has been possible to discover the gene involved. For example, if the LDLR pathway performance is compromised by a genetic defect in genes codifying one of the proteins, for example, LDLR or LDLRAP1, it can result in lipid disorders as Familial Hypercholesterolaemia (FH) or Autosomal Recessive Hypercholesterolaemia (ARH), respectively. The fundamental biological and cholesterol regulatory mechanisms related with cholesterol have being elucidated with the study of FH.

Familial Hypercholesterolaemia (FH) is a common autosomal dominant genetic disease clinically characterised by high levels of low-density lipoprotein cholesterol (LDL-C). LDL-C deposits in the tissues leads to the accumulation in tendons and arteries causing external manifestations, such as tendinous xanthomas and corneal arcus. [27] More importantly, LDL-C deposits in arteries can lead to premature atherosclerosis and increasing risk of premature CHD. [28]

FH exists in two forms: heterozygous (heFH) and homozygous (hoFH) form. HeFH is more common and less severe with a frequency, in most European countries, between 1/200 and 1/500; FH is very rare in the homozygous form, with a frequency of 1:300,000 to 1:1,000,000 in the general population. [16, 29]

1.3.1 Molecular Pathways That Cause Familial Hypercholesterolaemia

FH most frequently results from loss-of-function mutations in the *LDLR* gene representing >90% of the FH cases worldwide. [30] Mutations in this gene can affect any domain of the LDLR protein and result in: impairment in binding to the LDL particle, failure to internalize into the cell after binding, alteration in the transport to Golgi or to the plasma membrane, alteration in the recycle mechanism or a complete absence of the *LDLR* gene. [31, 32] Defects resulting from *LDLR* mutations lead to diminished catabolism of LDL-C, resulting in elevated plasma levels.

Mutations in *APOB* or *PCSK9* genes are also associated with FH, but they are less frequent. [31] A mutation in the *APOB* gene disables LDL-C from binding to the LDLR and leads to elevated LDL-C in the circulation. [33] PCSK9 is involved in the regulation of LDLR, targeting the LDLR for lysosomal degradation. [24] Loss-of-function mutations in this gene increase the number of LDLR on the cell surface which decrease the LDL-C levels, causing hypocholesterolaemia. [34] For this, recent studies are focusing on the potential of PCSK9-inhibiting compounds as a therapeutic target for dyslipidaemias. [35-36] On the other and, gain-of-function mutations in the *PCSK9* gene cause hypercholesterolaemia through higher activity of the LDLR-degrading function of PCSK9 with consequently increased plasma LDL-C plasma levels. [37-40]

The clinical phenotype resulting from these mutations is variable with *APOB* mutations being the least severe of the three. [41]

The distribution pattern of *APOE* polymorphisms affects the affinity to lipoprotein receptors and, consequently, the clearance of dietary fat from the blood. [42] E2, E3 and E4 are common protein isoforms encoded by three different alleles, ε2, ε3, and ε4. [43, 44] Two SNPs determine these isoforms: c.388C>T and c.526C>T. ApoE from VLDL, chylomicrons, and chylomicron remnants bind to specific receptor cells in the liver. Carriers of the ε2 allele are less efficient in transferring VLDLs and chylomicrons from the blood plasma to the liver due to its binding properties. [42] By contrast, carriers of the ε3 and ε4 alleles are more efficient in this process. While apo E4 and E3 bind with approximately equal affinity to lipoprotein receptors, apo E2 binds with less than 2 percent being associated with low cholesterol catabolism and hypertriglyceridemia in heterozygotes, since it can impair triglyceride hydrolysis, and type III hyperlipoproteinemia in homozygotes. [45]

The extremely rare recessive form of hypercholesterolaemia is ARH, caused by mutations in the *LDLRAP1* gene. [46] In ARH, the internalization of the ligand-receptor complex cannot occur resulting in impairment of LDL-C catabolism and increased LDL-C levels. [47]

1.3.1.1 LDL Receptor Gene and Protein

The *LDLR* gene, located on the distal short arm of chromosome 19, is comprised of 18 exons and 17 introns. [48] This gene encodes the LDLR, a cell surface glycoprotein, responsible for the binding and uptake of plasma LDL particles and plays a critical role in maintaining cellular cholesterol homeostasis. [49] It is synthesized in the rough endoplasmic reticulum (ER), processed in the Golgi apparatus, reaching the mature form, and transported to the cell surface (see Figure 1.1). [21] Despite the ubiquitous expression of LDLR, it is largely produced by the liver and expressed on the surface of hepatocytes. [2]

The 18 exons of *LDLR* gene encode five structural domains in the protein: three extracellular and two intracellular domains (see Figure 1.2). [50] Exon 1 codes for the signal sequence that directs the receptor from ribosomes to the ER membrane. Exons 2-6 encode the ligand binding domain, containing the binding for apoB, present in LDL, and apoE, present in VLDL and chylomicrons. The second domain is encoded by exons 7-14 and shares homology with a portion of the extracellular domain of the Epidermal Growth Factor (EGF) precursor. This domain is involved in the release of bound lipoproteins at low pH in the endosome. [22] Exon 15 encodes a domain that serves as an attachment site for O-linked carbohydrate chains. [51] The transmembrane domain is encoded by exon 16 and the 5'-end of

exon 17, and anchors the protein to the cell membrane. The last protein domain is a COOH-terminal cytoplasmatic tail encoded by the rest of exon 17 and exon 18. This domain is responsible for clustering the receptor in coated pits, allowing the internalization by receptor mediated endocytosis. [51, 22]

Changes in the codifying sequence of *LDLR* might cause serious impact at the protein level and, consequently, in the LDLR pathway leading, eventually, to increase or decrease of cholesterol levels in circulation.

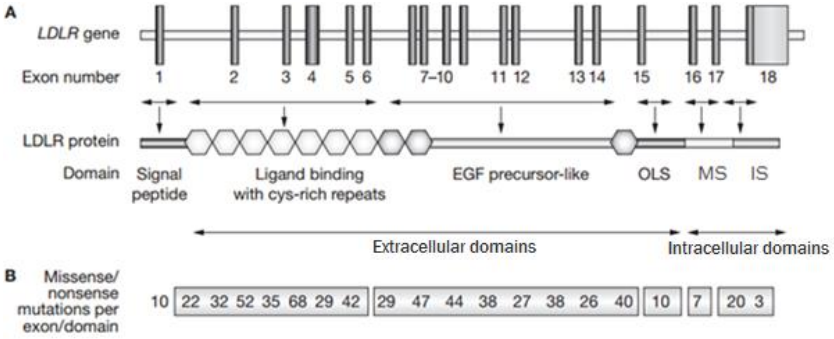

Figure 1.2 The *LDLR* gene and protein. (A) The *LDLR* gene: exons are shown as vertical dark bars, numbered underneath. Single exons or groups of exons (indicated by horizontal arrows) encode the different domains of the LDL receptor protein: the ligand binding domain, the EGF precursor-like domain, the O-linked sugars domain (OS), the membrane spanning domain (MS) and the internalization signal domain. (IS) (B) The number of point mutations in each exon or domain (boxed) that have been found in patients with familial hypercholesterolaemia worldwide. Adapted from [41]

1.3.2 *LDLR* Variants

Mutations in the different domains have distinct impact on LDLR structure and function. [2] Nevertheless, the simple detection of a variation in the coding sequence of FH-causing genes does not confirm the molecular diagnosis of FH. [52]

Functionally, the *LDLR* mutations have been classified into five classes: null (produce no detectable protein), transport-defective (complete or partial failure in LDLR transport between the ER and the Golgi apparatus), binding-defective (the receptor in the cell surface fail to bind LDL), internalization-defective (receptors are unable to cluster in clathrin-coated pits and thus do not internalize LDL) and recycling-defective (LDLR do not release the ligands in the endosome and cannot be recycled to the cell surface). [48]

Some variants can be promptly classified as pathogenic if they alter the correct synthesis/structure of the protein, usually translated in a truncated protein, without function, that would possibly be degraded by the cell. These variants include nonsense mutations leading to the occurrence of a premature stop codon, large rearrangements and insertions or deletions causing frameshift. [32] The pathogenicity of missense variants (the most common FH-causing mutations), deletions or insertions in frame, synonymous variants and intronic variants with a possible effect on splicing should be assessed with functional studies for an accurate FH genetic diagnosis. [53, 54]

1.3.3 Functional Studies

Although only few variants have been described in *APOB* and *PCSK9*, more than 1700 different alterations in the *LDLR* gene have been described worldwide. (http://www.ucl.ac.uk/ldlr/LOVDv.1.1.0 and http://www.hgmd.cf.ac.uk/ac/index.php) However, the functional studies for the majority of these variants, have not been performed, representing a serious problem for FH diagnosis. [55, 56] Only patients identified with variants previously described as pathogenic would have a definite diagnosis of FH, as the variants found can justify and confirm the clinical diagnosis.

Nonsense mutations and large rearrangements, usually, do not require a proof of their pathogenicity due to their evident effect in the protein. For missense and splicing alterations, the assessment by functional studies of the effect in the protein is mandatory for the genetic diagnosis of FH.

In the scope of the Portuguese FH Study, 44 functional studies have been performed.

1.3.3.1 Missense Variants

The human genome contains frequent single-basepair variants, referred as single nucleotide polymorphisms (SNPs) that may or may not cause a genetic disease. A missense variant is a point mutation that occurs in protein coding regions in the genome resulting in an amino acid substitution at the protein. It might affect gene function through their effect on the structure, folding or stability of the protein product. [57] The effect of a missense alteration also depends on the evolutionary conservation of an amino acid or nucleotide, the location and context within the protein sequence. [58] Consequently, the possible disease-association of missense variants is difficult to predict. Several *in silico* prediction software are available to predict the impact of a missense alteration. [58] However, most tools tend to have low specificity, overpredicting the missense alterations as deleterious. [59] This emphasizes the importance of functional studies to assess their real effect on the protein.

During the Portuguese FH Study, 31 missense disease causing variants were found, being the most common cause of FH.

1.3.3.2 Splicing Variants

Generally, synonymous variants are assumed to be "silent". However, they may disrupt normal splicing or mRNA folding and stability, which may affect normal peptide synthesis. They can also affect the codon usage leading to differences in codon adaptation to tRNA pools and, consequently, influencing the speed of translation elongation or transcription/gene expression. The same thought can be applied to missense mutations in introns which may contain splicing regulatory regions. Variations in these elements, including splice donor (GT), acceptor sites (AG) and exon splice enhancer (ESE) elements, can result in complete or partial skipping of the exon, retention of the intron or the introduction or activation of a new splice site. [60] Nevertheless, nucleotide substitutions in less conserved positions can cause splicing defect in some but not all cases. Accordingly, RNA studies are required to determine if an incorrect splicing effect is present.

Approximately 258 different mutations have been identified in the scope of the Portuguese FH study, 13 of which cause splicing alterations. Characterization of these splicing variants and quantification of transcripts can lead to the identification of new drug targets and a more precise medicine.

1.3.4 Diagnosis of Familial Hypercholesterolaemia

Familial hypercholesterolaemia is underdiagnosed and undertreated worldwide, particularly among children. [61] FH patients present high levels of plasma cholesterol since birth and develop premature CHD. Therefore, an early diagnosis of FH is crucial to prevent the consequent morbidity and mortality from premature CHD in these patients. FH diagnosis comprise a combination of family history of CHD, clinical signs as xanthomas and cholesterol levels. Secondary causes as diabetes, hypothyroidism, hepatic and renal diseases, should be excluded for the diagnosis. [21] There are different FH clinical criteria to diagnose index cases. The most commonly used are the USMEDPED (Make Early Diagnosis to Prevent Early Death), [62] the UK (Simon Broome) [63] and the Dutch Lipid Clinic. [64] Nevertheless, only genetic testing may give a definite diagnosis of FH by detection of a pathogenic variant.

1.3.5 Treatment

FH patients have increased LDL-C levels over a lifetime and, consequently, develop CHD at significantly earlier ages and at a greater frequency than the general population. [65] To prevent this, treatment should be implemented immediately once the diagnosis of FH is achieved. Reducing cholesterol levels and controlling risk factors as smoking, diet and physical activity are the initial treatment strategies. [66]

Several lipid-lowering drugs are currently available. HMG-CoA reductase inhibitors, known as statins, inhibit the production of endogenous cholesterol and increase the expression of LDLR thereby increasing LDL uptake and catabolism. This results in the reduction of LDL-C between 20-45%, depending on the dosage. [67] Nonetheless, statins do not lower LDL significantly in hoFH patients, who have null variants in both copies of the *LDLR*. [68]

Ezetimibe, a drug that inhibits cholesterol absorption, reduces the amounts of dietary cholesterol that reach the liver. [69] Used as monotherapy, ezetimibe cause a reduction of almost 20% in LDL-C. [70] However, it can also be coadministraded with statins which will decrease LDL cholesterol by 60–70%. [29] Since ezetimibe is usually better tolerated, it may become the first choice for heFH patients who do not tolerate high doses of statins. [64]

Recently, plant sterols and stanols have been demonstrated to exert a beneficial effect on lowering LDL-C concentrations through inhibition of intestinal cholesterol absorption, even in the presence of diets with low cholesterol content. [71] The administration of plant sterols and stanols is common in heFH children and reduce LDL-C by 10 and 15%, respectively. [72, 73]

Plasma levels in hoFH patients or in heFH patients resistant to lipid-lowering drugs can only be lowered with LDL apheresis, an extracorporeal procedure to remove the LDL-C from the blood.

New LDL lowering drugs have been discovered with the advances in genetic-based pharmacology: monoclonal antibodies targeting PCSK9, anti-sense oligonucleotides targeting apoB and cholesteryl ester transfer protein inhibitors. [29] Nevertheless, further studies are required to determine the long-term safety of these therapeutic measures and their efficacy in preventing CHD. [35]

1.4 Portuguese FH Study

In 1998, the World Health Organization (WHO) recommended universal screening for FH. Hence, in 1999, the Portuguese FH study was established at the National Institute of Health. This study was designed to identify the genetic cause of hypercholesterolaemia in individuals with a clinical diagnosis of FH, cascade screening in relatives of the affected index patients and to determine the prevalence and distribution of FH in Portugal. [74] Index patients are included in this study using an adapted Simon Broome (SB) criteria. [56]

In the last 16 years, a genetic defect was identified in 663 patients, representing 3.32% of the cases estimated to exist in Portugal. The implementation of the molecular study of this disorder in Portugal, promotes the early identification, in these patients and their relatives, leading to a correct counselling and decreasing their cardiovascular risk.

This study implies a biochemical and a genetic diagnosis of FH, performed in four phases and an optional fifth phase, as schematically represented in Figure 1.3.

Biochemical Study	
Lipid Profile	Total cholesterol; LDL-C; HDL-C; Triglycerides; Apolipoprotein B; Apolipoprotein A1; Lipoprotein (a)
Molecular Study	
Phase I	DNA extraction. Screening for the common mutations in *APOB* gene, analysis of *LDLR* gene and exon 4 of *APOE* gene
Phase II	Identification of large rearrangements in the *LDLR* gene using MLPA technique
Phase III	Screening of *PCSK9* gene (only performed if no mutation was found in phases I and II)
Phase IV	*APOB* gene: study of promoter, all exons and flanking regions in selected patients (only performed if no mutation was found in phases I, II and III)
Phase V	Functional *in vitro* studies of *LDLR*, *APOB* or *PCSK9* alterations identified with unknown pathogenicity

Figure 1.3 Phases of molecular study in the Portuguese FH study. Adapted from [56]

When a putative disease-causing variant is identified in an index case, a cascade screening is performed if samples of relatives are available. The cascade screening has been proved to be the most cost-effective method of identification and has an important role in identifying young patients. [75] Early identification of FH and the implementation of preventive measures and correct treatment can prevent premature CHD.

1.5 Aim of the Present Work

Although the Portuguese FH Study has been implemented in Portugal in 1999 and more than 800 families have been enrolled [56], only 3.32% of the cases estimated to exist in Portugal were identified. The extent of underdiagnosis and undertreatment of individuals in the Portuguese population with FH is alarming.

In order to improve the identification of individuals at risk, the main aim of this project is to perform the molecular identification of genetic variants causing dyslipidaemia in *LDLR, APOB* and *APOE* genes, in patients referred to the Portuguese FH Study in 2015/2016 with a clinical diagnosis of FH. Different *in silico* analysis and functional studies of putative splicing variants will be performed to achieve this aim.

2 METHODS

2.1 Patients recruitment

2.1.1 Study Population

Patients were recruited, all over the country, for the Portuguese Familial Hypercholesterolaemia Study, whose protocol and database have been approved by the National Institute of Health Ethics Committee and the National Data Protection Commission, respectively. A total of 60 index patients (35 adults and 25 children) and 28 affected and unaffected relatives where included in this study. Written informed consent was obtained from all participants before their inclusion in the study. Index patients were selected following an adaptation of the Simon Broome criteria for FH. [76] The Simon Broome criteria categorises familial hypercholesterolaemia as "definite" or "possible". "Definite" familial hypercholesterolaemia is defined as total cholesterol (TC) concentration >290 mg/dL or LDL-C concentration >190 mg/dL for adults and TC concentration >260 mg/dL or LDL-C concentration >155 mg/dL for children under 16, plus tendon xanthomas in the patient or a first or second degree relative and genetic evidence of mutation in *LDLR* or *APOB* genes. A "possible" diagnosis of familial hypercholesterolaemia requires cholesterol above these values, plus a family history of either a myocardial infarction before age 50 in a second degree relative or before age 60 in a first degree relative or a raised TC concentration >290 mg/dL in a first or second degree relative. More recently, newly determined cut-off points for lipid biomarkers as apoB/apoA1 ratio ≥0.68 have been conjugated with the Simon Broome criteria to improve children identification, especially trough clinical differentiation between monogenic and polygenic dyslipidaemia. [77]

2.1.2 Blood Samples Collection

For each index case and respective relatives, fasting blood samples were collected in order to perform DNA extraction (3 x 2,7 mL in EDTA tubes) and biochemical determination (7,5 mL in serum tube). A confidential identification number was assigned for each sample and all the information concerning the patients was registered in a confidential database, according to legal requirements.

2.1.3 Biochemical Characterization

The biochemical parameters, including total cholesterol (TC), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), triglycerides (TG), apolipoprotein AI (ApoAI), apolipoprotein B (ApoB) and lipoprotein (a) (Lp(a)), were determined at the Unidade Laboratorial Integrada at INSA, using an autoanaliser Cobas Integra 400 plus (Roche) by enzymatic, colorimetric and immunoturbidimetric methods.

2.2 Molecular Biological Techniques

2.2.1 Genomic DNA extraction

Genomic DNA was isolated from peripheral blood EDTA samples, using an adaptation of the protocol described in D.K.Lahiri *et al.* (1991). For index cases, DNA extraction was performed for all collected tubes, in independent days, providing different DNA samples for diagnosis confirmation. The proportion of each reagent to mL of blood is presented in Appendix I, Table A I.1. Whole blood was homogenised and transferred into a 15 mL falcon tube at which was added equal volume of TKM X-100 (low salt buffer containing 10 mM Tris-HCl pH 7.6, 10 mM $MgC1_2$ and 2 mM EDTA and 25 mL of Triton X- 100/L). After mixing by inversion, IGEPAL was also added to lyse the cells and the blood was mixed until total solubilisation. The tubes were centrifuged for 10 min at 2200 rpm, at room temperature (RT) (centrifuge 5810 R, Eppendorf). The supernatant was poured off and the pellet was washed in TKM1 buffer (TKM-X100 without the Triton-X 100) and centrifuged for 10 min at 1600

rpm, at RT. After repeating this step once, the pellet was ressuspended in TKM2 (high salt buffer containing 10 mM Tris-HC1 pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 0.4 M NaCl and 2 mM EDTA). After addition of SDS 10%, the suspension was mixed and incubated for 10 min at 55°C for protein denaturation. Afterward, the whole content was transferred to a 2 mL Eppendorf tube and NaCl 5M (60 µL for mL of blood) was added for protein precipitation. Once centrifuged for 20 min at 13200 rpm, at RT, in microcentrifuge, all the supernatant was transferred to a clean falcon tube and absolute ethanol (2.3 mL for 1 mL of blood) was added and gently mixed by inversion, in order to denature and precipitate the DNA. The DNA fibrils were removed with a loop and washed with 70% ethanol. When dried, the DNA was transferred into an Eppendorf with TE (10 mM Tris-HCL, 1 mM EDTA ph 8.0).

DNA purity (A260 and A280) and concentration were determined using the NanoDrop ND-1000 (Thermo Scientific). The quality of DNA was assessed by agarose gel electrophoresis, using 1% agarose gel (w/v), prepared with TBE buffer 1x (Invitrogen), with 0.002% of SYBR Safe DNA gel stain (Invitrogen) and mixing 1 µL of genomic DNA diluted in bidistilled water with the gel loading die (bromophenol blue) to a final volume of 10 µL. The electrophoresis was performed in a Bio-Rad Power Pac 3000 equipment for 40 min at 90 Volt, in TBE 1x, and the gel was visualized in a Safe Imager™ blue light transilluminator (Invitrogen).

2.2.2 DNA analysis

The promoter region and the 18 exons plus flanking regions of the *LDLR* gene were amplified from genomic DNA by polymerase chain reaction (PCR) and screened for sequence alterations using Sanger sequencing. Screening for the common mutations in the *APOB* and *APOE* gene was performed by PCR amplification and sequencing fragments of exons 26 and 29 for *APOB* and exon 4 for *APOE*. A list with the primers used and the distinct annealing temperatures for each exon, as well as the protocol used to prepare each PCR mix, are disclosed in Appendix I, table A I.2 and A I.3, respectively. The PCR was performed in a T3000 thermocycler (Biometra) using the following cycle conditions: initial denaturation for 3 min at 95ºC; 35 cycles of three steps: denaturation for 45 sec at 94ºC, annealing for 30 sec at primer specific annealing temperature, and elongation for 1 min at 72ºC; and final extension for 30 min at 72ºC. The PCR products were stored at 4ºC.

All PCR products were assessed by an agarose gel electrophoresis, using 1,5% agarose gel (w/v), prepared with TBE buffer 1x (Invitrogen), with 0.002% of SYBR Safe DNA gel stain (Invitrogen) and mixing 5 µL of PCR product with the gel loading dye (bromophenol blue) to a final volume of 10 µL. The 100bp DNA ladder was used to confirm the approximate size of the PCR products. The electrophoresis was performed in a Bio-Rad Power Pac 3000 equipment for 40 min at 90 Volt, in TBE 1x, and the gel was visualized in a Safe Imager™ blue light transilluminator (Invitrogen).

2.2.3 Automated sequencing

Before Sanger sequencing, PCR products were purified to remove the excess of primers and dNTPs. The enzymatic digestion was performed using two hydrolytic enzymes: Exonuclease I and Shrimp Alkaline Phosphatase (SAP), combined in a commercial product named ExoStar (IllustraTM ExoStarTM, GE lifesciences). To 2,5 µL of PCR product, 1 µL of ExoStar was added in a reaction tube and incubated for 15 min at 37ºC (enzyme optimum temperature) and for 15 min at 80ºC for enzyme inactivation. Purification products were stored at 4ºC until further use. The sequencing reaction was prepared as follows: 1 µL of primer at 2 ρmol/ µL, 1 or 2 µL of BigDye (Terminator Cycle Sequencing Ready reaction kit, Applied Biosystems), depending on fragment length, and bidistilled water were added in a reaction tube to a final volume of 9 µL. At last, 1 µL of purified PCR product was added. The sequencing reaction was performed in a T3000 thermocycler (Biometra) using the following cycle conditions: initial denaturation for 30 sec at 96ºC; 25 cycles of three steps: denaturation for 10 sec at 96ºC, annealing for 5 sec at 50ºC and elongation for 4 min at 60ºC. Sequencing products were stored at

4ºC. The resulting products were sequenced at the Unidade de Tecnologia e Informação (UTI – INSA) (3130xl Genetic Analyser, Applied Biosystems). The subsequent ABI sequence files were analysed with Staden Package software (version 2.0). Gene mutation nomenclature used in this study follows the recommendations of Human Genome Variation Society (www.hgvs.org). The reference sequences used were *APOB*: NM_000384.2; *LDLR*: NM_000527.4; *APOE*: NM_000041, where +1 is the A of the ATG translation initiation codon of the coding DNA. [78] Variant nomenclature was revised using the program Mutalyzer (https://mutalyzer.nl). Whenever an alteration was found, all PCR, assessment and sequencing protocols were repeated in a second independent sample of the index case, and the molecular study was also performed for the relatives, when samples were available, for confirmation and co-segregation studies.

2.2.4 Multiplex Ligation-dependent Probe Amplification (MLPA)

The SALSA® MLPA® kit (probemix P062-C2 LDLR, MRC-Holland, The Netherlands) was used, according to the manufacturer's instruction, to search for large rearrangements (duplications or deletions) in the *LDLR* gene. The MLPA protocol is divided in four major steps: sample DNA denaturation, probes hybridization, probe ligation and PCR reaction for amplification of ligated probes. Fragments were separated by capillary electrophoresis, performed by Unidade de Tecnologia e Informação (UTI – INSA) (3130xl Genetic Analyser, Applied Biosystems) and the data was analysed using Coffalyser – MLPA analysis tool (MRC-Holland, The Netherlands).

2.2.5 Study of variants affecting mRNA splicing

2.2.5.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Peripheral Blood Mononuclear Cells (PBMC) were isolated from fresh blood collected in CPT tubes (cell preparation tube with sodium citrate, BD Vacutainer®) by centrifugation for 30 min at 2800 rpm, at room temperature (18ºC) (separation of PBMC must occur during the first 2 h after collection). After PBMC resuspension trough inversion of CPT tube, the upper layer containing plasma and PBMC was transferred to a 15 mL falcon tube. The PBMC were obtained by centrifugation for 10 min at 1600 rpm, 4ºC. The plasma was separated and stored in 2 mL eppendorfs at -80ºC. The mononuclear cells (pellet) were then washed with 1 mL of PBS 1X: 1 µL of this cellular suspension was added to 9 µL of Trypan Blue (1:10 diluted) and used for cell count, applying the 10 µL in a Neubauer counting chamber; the remaining suspension was centrifuged for 10 min at 3000 rpm, 4ºC. The resulted pellet was resuspended in 350 µL ($<5x10^6$ cells) to 600 µL ($\geq 5x10^6$ to $1x10^7$ cells) of RLT Buffer (Quiagen) (1% Mercaptoethanol) with a syringe (27G) to perform cellular lysis. The lysate was centrifuged, in a QIAShredder mini column, for 2 min at 13200 rpm, 4ºC. The tubes were stored at -80ºC until RNA extraction.

2.2.5.2 RNA extraction

Total RNA was extracted with the RNeasy® Mini Kit (Qiagen). After thawing the samples, equal volume of ethanol 70% (V/V) (-20ºC) was added to the lysate and then homogenised. The total volume was transferred to an RNeasy column and centrifuged for 15 sec at 11000 rpm at 18ºC. The eluted volume was poured off. Depending on lysate volume, 350 µL to 500 µL of ethanol 70% (V/V) were added to the column and centrifuged as above. The column was transferred to a new collector tube and 350 µL of RW1 buffer solution was applied right in the centre of the column. After gently mixing by inversion, the tube was centrifuged as above and the volume in the collector tube was discarded. For one sample, the Mix DNase I was prepared adding 70 µL of RDD buffer to 10 µL of DNase I and gently mixing. The 80 µL of this mix were directly applied in the centre of the membrane and incubated at room temperature (18ºC - 30ºC) for 15 min. To remove the DNase I, 350 µL of buffer RW1 solution were added and the tubes were centrifuged for 15 sec at 11000 rpm, 18ºC. The eluted volume was poured

off, 500 µL of RPE buffer solution were mixed in and the centrifugation was performed as in the previous step. This step was repeated once and then a new centrifugation in the same conditions was performed to remove all RPE buffer. The column was transferred to a new Eppendorf of 1.5 mL and 50 µL of RNase free water were applied in the centre of the membrane. The tube was centrifuged for 1 min at 13000 rpm at 4ºC to elute the RNA. The column was discarded and 6 to 10 µL were removed to an Eppendorf and used for quantitative and qualitative analysis of RNA. The remaining volume was distributed in aliquots and stored at -80ºC.

The RNA purity (A260 and A280) and concentration were determined using the NanoDrop ND-1000 (Thermo Scientific) and quality was assessed by agarose gel electrophoresis, using 1% agarose gel (w/v). The gel was prepared with TBE buffer 1x (Invitrogen), with 0.002% of SYBR Safe DNA gel stain (Invitrogen) and mixing 5 µL of RNA with the gel loading dye (bromophenol blue) to a final volume of 10 µL. The electrophoresis was performed in a Bio-Rad Power Pac 3000 equipment for 40 min at 90 Volt, in TBE 1x, and the gel was visualized in a Safe Imager™ blue light transilluminator (Invitrogen).

2.2.5.3 Reverse transcriptase (RT) reaction and cDNA analysis

To prepare cDNA, 1µg of RNA was reverse transcribed with High capacity RNA-to-cDNA kit from Applied Biosystems, according to the manufacturer's instruction. For the RT mix, 10 µL of RT Buffer 2X, 1 µL of enzyme 20X, 1 µg of RNA and DEPC water were added in a reaction tube to a final volume of 20 µL. In each reaction, no template control (NTC) and no enzyme control (NEC) were added, to ensure that no contaminants were present in the reaction and to determine that any amplification that occurs in the sample was derived from the synthesised cDNA and not from genomic DNA or other amplicon contamination. cDNAs were stored at -30ºC.

PCR was performed to amplify *LDLR* coding sequences. Regions of interest were amplified with specific primers designed to evaluate the effect on cDNA of each splicing variant found. A list with the primers used, the annealing temperatures and the protocol used to prepare each PCR mix, are disclosed in Appendix I, table A I.4 and A I.5. The PCR was performed in a T3000 thermocycler (Biometra) using the following cycle conditions: initial denaturation for 2min at 94ºC; 38 cycles of two steps: denaturation for 45 sec at 95ºC, annealing for 1min 45 sec at primer specific annealing temperature followed by elongation for 7 min at 65ºC and final extension for 30 min at 4ºC. The PCR products were stored at 4ºC.

Specific detection of each transcript was accessed by an agarose gel electrophoresis, using 3% agarose gel (w/v) with NuSieve™ 3:1 Agarose. The gel was prepared with TBE buffer 1x (Invitrogen), with 0.002% of SYBR Safe DNA gel stain (Invitrogen) and mixing 8 µL of cDNA product with the gel loading dye (bromophenol blue) to a final volume of 10 µL. The pUC18 DNA Hae III ladder was used to confirm the approximate size of the PCR products. The electrophoresis was performed in a Bio-Rad Power Pac 3000 equipment for 1h at 50 Volt, in TBE 1x, and the gel was visualized in a Safe Imager™ blue light transilluminator (Invitrogen).

2.2.6 *In silico* Analysis

When any variant was found in *LDLR*, *APOB* or *APOE* genes, the predicted effects were assessed using the following open access software: Polymorphism Phenotyping (PolyPhen-2) [59], Sorting Intolerant From Tolerant (SIFT) [79], Protein Variation Effect Analyzer (PROVEAN) [80] and MutationTaster [81] for prediction of single nucleotide substitutions. SIFT takes into account evolutionary conservation through the use of sequence alignments, while PROVEAN, MutationTaster and PolyPhen-2 base their predictions in protein structure/function and evolutionary conservation. Human Splicing Finder (HSF) (http://www.umd.be/HSF3/HSF.html) [82], the Nearest-neighbor

Secondary Structure Prediction (NNSSP) (http://www.fruitfly.org/seq_tools/splice.html) [83] and the FSPLICE (http://linux1.softberry.com/) tools were used for prediction of splicing defects. HSF uses position-dependent logic, identifying exonic and intronic motifs. NNSSP is based in neural networks combining a sequence similarity matrix with a local structural environment scoring scheme for predicting protein secondary structure and FSPLICE (http://linux1.softberry.com/) bases its predictions on weight matrices model, which consider the importance of the presence of a determinate nucleotide in a specific position. Mutation Taster also predicts a phyloP score, a measurement of evolutionary conservation; thus the higher the score, the stronger is the evolutionary conservation for that specific nucleotide. Variants were classified as pathogenic, likely pathogenic, benign and likely benign according to the American College of Medical Genetics (ACMG) recommendations. [58] When the classification is contradictory or if that is not enough points for classification in either category, the variant is classified as variant of unknown significance (VUS).

3 RESULTS

3.1 Molecular Diagnosis

A group of 60 unrelated individuals were studied, 25 children and 35 adults. A total of 28 relatives, affected and unaffected, were also studied. A summary of biochemical characterization of patients presenting values before medication and clinical characterization of all patients is presented in Table 3.1. A more detailed characterization including information as age, cardiovascular events and medication is presented in Appendix II, Table A II.1.

Table 3.1 Summary of clinical and biochemical characterization.

	TC	LDL-C	HDL-C	TG	ApoB/ ApoA1	On medication	CHD
Children	292 mg/dL (n=23)	214 mg/dL (n=23)	58 mg/dL (n=23)	92 mg/dL (n=23)	0.94 (n=23)	52% (n=25)	0% (n=25)
Adults	346 mg/dL (n=19)	259 mg/dL (n=19)	55 mg/dL (n=19)	156 mg/dL (n=19)	0.98 (n=19)	85% (n=34)	49% (n=35)

3.1.1 Molecular Study of *LDLR*, *APOB* and *APOE* Genes

The promotor region, 18 exons of *LDLR* and adjacent regions, as well as part of exons 26 and 29 of *APOB* and exon 4 of *APOE* were analysed for all index patients. No homozygous patients were found in this study. However, 24 individuals were identified as heterozygous for 18 different variants. These include 12 missense alterations (1 with unknown effect and 11 causing an aminoacid change), 3 nonsense alterations resulting in a premature stop codon and 3 putative splice alterations (see Table 3.2).

All patients included in the study had severe hypercholesterolaemia and family history of high levels of cholesterol, 17 had cardiovascular disease and 22 presented family history of CHD. However, it was not possible to identify a clear relationship between phenotype and family history with the presence or not of a pathogenic variant, supporting the importance of the molecular confirmation of a clinical diagnosis.

Of the 24 index patients with a possible mutation, 12 were children and the other 12 were adults (see figure 3.1).

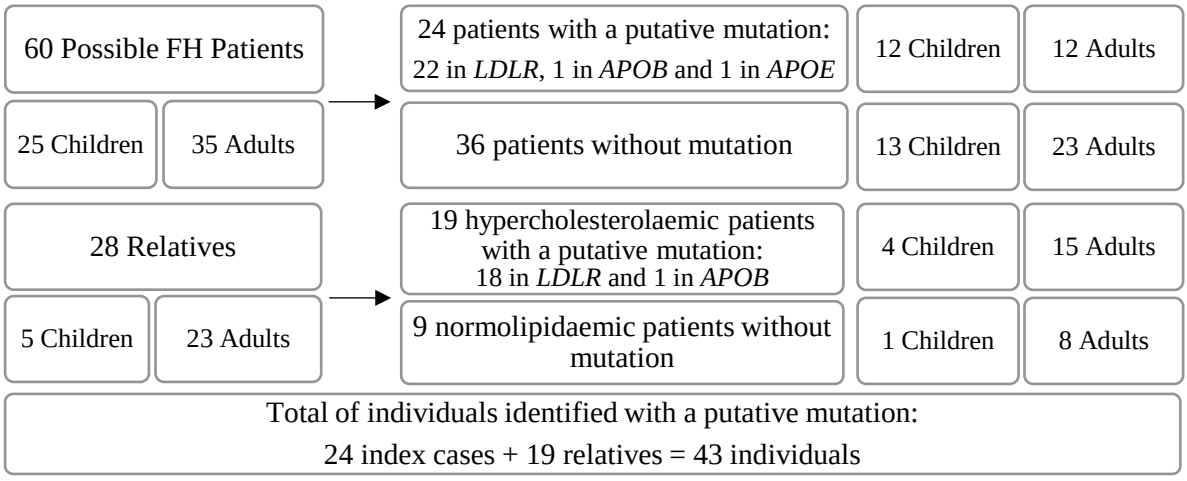

Figure 3.1 Summary of molecular diagnosis results.

Table 3.2 Variants in *LDLR, APOB* and *APOE* genes identified in studied index cases.

Patient	Alteration					Described by	Functional studies	Coseg
	Location	Nucleotide Change	Protein	Domain	Effect on protein			
18	LDLR Exon 1	c.1 A>C	p.(?)	Signal sequence	Unknown	[84-86]	NP	2/2;0/0
12	LDLR Exon 3	c.261G>A	p.(Trp87*)	Ligand binding	PSC	[86]	Yes	3/3;0/0
28	LDLR Exon 4	c.551G>A	p.(Cys184Tyr)	Ligand binding	aa change	[74,87-90]	Yes	1/1;0/0
1,8,20,57	LDLR Exon 4	c.670G>A	p.(Asp224Asn)	Ligand binding	aa change	[48,91-92]	Yes	7/7;0/3
39	LDLR Exon 4	c.693C>G	p.(Cys231Trp)	Ligand binding	aa change	[93-95]	NP	1/1;0/0
10	LDLR Exon 6	c.862G>A	p.(Glu288Lys)	Ligand binding	aa change	[92,96]	Yes	2/2;0/0
33	LDLR Intron 6	c.941-2A>C	p.(?)	-	Splicing	[95,97]	NP	1/1;0/0
14,26	LDLR Intron 7	c.1060+1G>A	p.Gly314_Glu353del	-	Skipping of exon 7	[74,98]	Yes	3/3;0/2
21,29	LDLR Exon 9	c.1216C>T	p.(Arg406Trp)	EGF percursor homology	aa change	[99,100]	Yes	4/4;0/1
34,50	LDLR Exon 9	c.1291G>A	p.(Ala431Thr)	EGF percursor homology	aa change	[90,95,100-104]	Yes	3/3;0/2
37	LDLR Exon 10	c.1374_1375del	p.(Arg458Serfs*8)	EGF percursor homology	PSC	[105]	NP	3/3;0/0
58	LDLR Exon 10	c.1432G>A	p.(Gly478Arg)	EGF percursor homology	aa change	[48,90,106-108]	Yes	1/1;0/0
6	LDLR Exon 13	c.1886del	p.(Phe629Serfs*36)	EGF percursor homology	PSC	[92]	NP	1/1;0/1
25	LDLR Exon 13	c.1897C>T	p.(Arg633Cys)	EGF percursor homology	aa change	[109-111]	NP	2/2;0/0
59	LDLR Exon 14	c.2054C>T	p.(Pro685Leu)	EGF percursor homology	aa change	[74,107,112-115]	Yes	1/1;0/0
5	LDLR Exon 16	c.2389G>A	p.Ala771Valfs*17	Membrane spanning	Skipping of exon 16/PSC	[90,97,107, 116,117]	Yes	4/4;0/0
19	APOB Exon 26	c.10580G>A	p.(Arg3527Gln)	Domain 6	aa change	[33,118-122]	Yes	2/2;0/0
53	APOE Exon 4	c.487C>T	p.(Arg163Cys)	α-helix H4	aa change	[123,124]	NP	1/1;0/0

NP, not performed. Coseg, co-segregation in studied families: variant carriers/total affected; variant carriers/total non-affected. PSC, premature stop codon. aa change, aminoacid change

Eighteen different variants were found: 16 in *LDLR* gene, 1 in *APOB* and 1 in *APOE*. Despite the fact that 11 of these variants have functional studies previously reported and 2 are nonsense variants, considered pathogenic due to the severity of the defect (premature protein termination), *in silico* predictions were performed for all of them (see Table 3.3). The confirmation of the clinical diagnosis was only achieved for 19 of 24 index patients (79%) since only 13 variants have proof of its pathogenicity.

The pathogenicity of the remaining 5 variants was not proven until now and so, definite diagnosis was not possible for the 5 index patients with these variants (21%): 2 children and 3 adults. In children, the 2 missense variants in *LDLR*, without functional studies, are classified as likely pathogenic following ACMG recommendations. In adults, 1 of the 3 variants is a missense variant in *LDLR* likely to be pathogenic, 1 is a putative splicing variant in *LDLR* classified as pathogenic but with no prove of its effect and the last one is a missense variant in *APOE* described in patients with other type of dyslipidaemia, classified by ACMG as VUS.

Considering all alterations found, the positive genetic rate was 48% in children and 34% in adults. These values decrease if only proved pathogenic mutations are considered (40% in children and 26% in adults). No mutation was found in 36 patients in the three studied genes, representing 60% of the index cases studied here.

Several variants detected in this study were first described in other populations (see Table 3.1), apart from variant c.1060+1G>A, first described in Portugal and already functionally characterized.

For index patients with an identified variant, the cascade screening was performed for their relatives if samples were available. A total of 28 relatives were studied allowing the identification of another 17 definite FH patients (4 children and 13 adults) and 2 possible FH patients.

3.1.1.1 *LDLR* Gene

Of the 16 variants found in *LDLR* gene, 12 are pathogenic: 10 were previously proved to be pathogenic by functional studies and 2 are nonsense variants considered pathogenic due to their effect (see Table 3.4). Concerning their location, the EGF precursor homology domain has the largest number of variants detected in this study, as shown in Figure 3.2. Two of the variants found in these patients are present in the noncoding sequence.

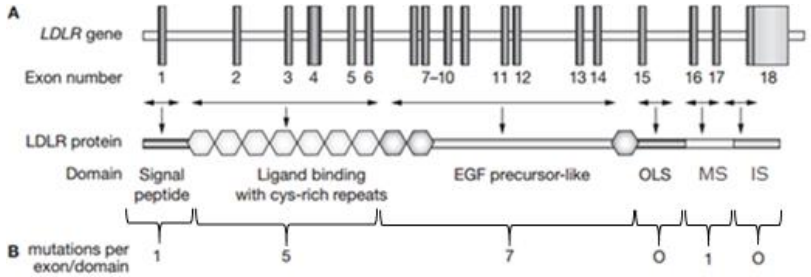

Figure 3.2 The *LDLR* gene and variants found in each domain. (A) The *LDLR* gene and protein domains: ligand binding domain, EGF precursor-like domain, O-linked sugars domain (OS), membrane spanning domain (MS) and internalization signal domain (IS). (B) The number of point mutations in each domain that have been found in patients with clinical diagnosis of familial hypercholesterolaemia during this study. Adapted from [41]

3.1.1.1.1 Missense Variants in *LDLR*

The majority of the variants identified in the studied patients are missense mutations localized in the EGF precursor homology and ligand binding domains. Only 7 of the 10 missense mutations found in *LDLR* gene were previously proved to be pathogenic.

The effect of c.1 A>C alteration is unknown. Since this variant occurs in the first codon of exon 1, corresponding to the ATG codon start, it is thought to be a missense variant without protein production. It is classified as probably pathogenic following ACMG recommendations and overall *in*

silico results in a non-concordant prediction. The remaining 9 alterations cause an aminoacid change in the protein and all of them are predicted to be pathogenic by overall *in silico* software packages. p.(Cys184Tyr), p.(Asp224Asn), p.(Cys231Trp) and p.(Glu288Lys) are variants in the exon 4 and 6 of *LDLR*, corresponding to the ligand binding domain of the protein. p.(Arg406Trp), p.(Ala431Thr), p.(Gly478Arg), p.(Agr633Cys), p.(Pro685Leu) occur in the EGF precursor homology domain. None of these missense mutations were predicted to affect splicing. According to ACMG classification, 4 of them are classified as pathogenic, 5 as likely pathogenic and 1 as VUS (see Table 3.3).

3.1.1.1.2 Nonsense Variants in *LDLR*

Three nonsense variants have been found: p.(Trp87*) results in a premature stop codon in the ligand binding domain; p.(Arg458Serfs*8) and p.(Phe629Ser fs*36) are frameshift mutations caused by a deletion changing the reading frame and resulting in a premature stop codon.

3.1.1.1.3 Splicing Variants in *LDLR*

Three putative splicing variants were found in the index patients analysed.

c.941-2A>C, p(?)

A blood sample for the RNA study was not available for the patient with this alteration. However, Sanger sequencing of patient's DNA revealed a deletion of the acceptor site of exon 7, probably causing retention of part or the entire intron 6. The variant is classified as pathogenic by overall *in silico* predictions with a reduction or complete absence of the acceptor site. ACMG also classifies it as pathogenic.

c.1060+1G>A, p.Gly314_Glu353del

Sanger sequencing of patients' DNA with this variant revealed a disruption of the donor site in intron 7. This is expected to cause total skipping of exon 7 (see Figure 3.3). The splice site scores showed a reduction from 100% for the natural donor site to 69 or 0% for the variant site. Blood samples from these patients were not available for the RNA study. However, it was possible to have a blood sample from another patient, with the same alteration, for the study of the mutation mechanism.

A fragment of mRNA encompassing the region encoding exons 5 to 9 was amplified and the agarose gel electrophoresis of the product revealed two fragments: the expected fragment of 497 bp and a smaller fragment of approximately 377 bp, lacking the exon 7, confirmed by Sanger sequence (see Figure 3.3).

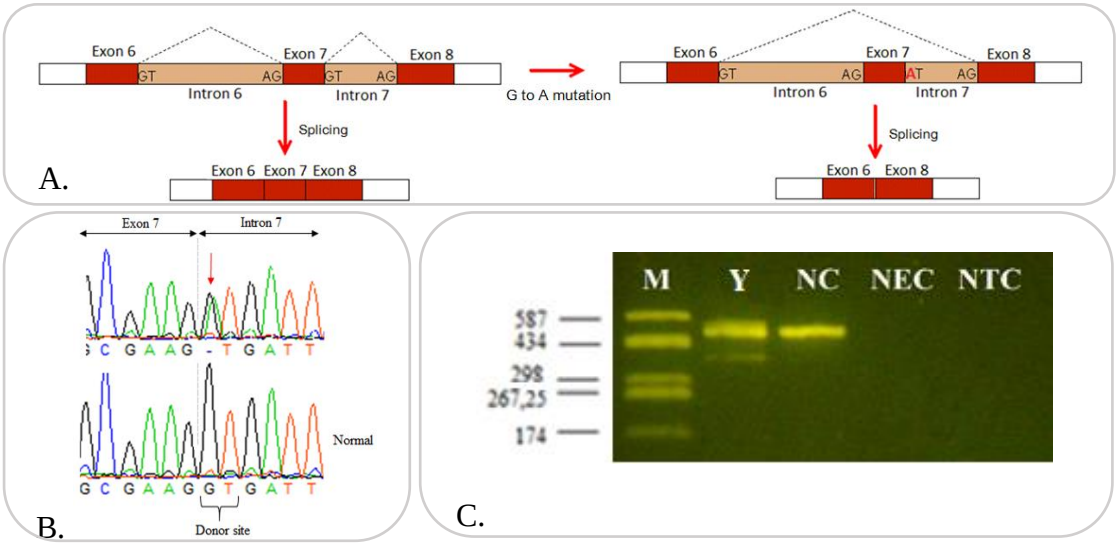

Figure 3.3 Functional study of c.1060+1G>A alteration. A. Schematic effect of c.1060+1G>A mutation in intron 7 of *LDLR* gene, resulting in exon 7 skipping. B. Comparison between DNA electropherograms of patient with c.1060+1G>A alteration and normolipidaemic control. C. Gel-electrophoresis of amplicons encompassing exons 5 to 9. One index case of the Portuguese FH Study with c.1060+1G>A alteration is shown (Y). A normolipidaemic individual was used as negative control (NC). No enzyme control (NEC) and no template control (NTC) were also included in the experiment. pUC18 DNA Hae III Digest was used as ladder (M). The size (in base pairs) of each fragment is indicated on the left side.

c.2389G>A, p.Ala771Valfs*17

Sanger sequencing of patients' DNA with this variant revealed the creation of a new acceptor site in the end of exon 16 (see Figure 3.4). Human Splicing Finder predicted a splice site score of 150% for the variant site when compared to the 100% of the normal acceptor site. On the other hand, FSPLICE predicted a considerable reduction (<88%) of the donor splice site score from 100% to 52% for the variant site.

A fragment of mRNA encompassing the region encoding exons 15 to 18 was amplified from samples of the index patient and three relatives by RT-PCR. The agarose gel electrophoresis of the product revealed two fragments: the expected fragment of 381 bp and a smaller fragment of approximately 303 bp, lacking the exon 16, confirmed by Sanger sequence (see Figure 3.4).

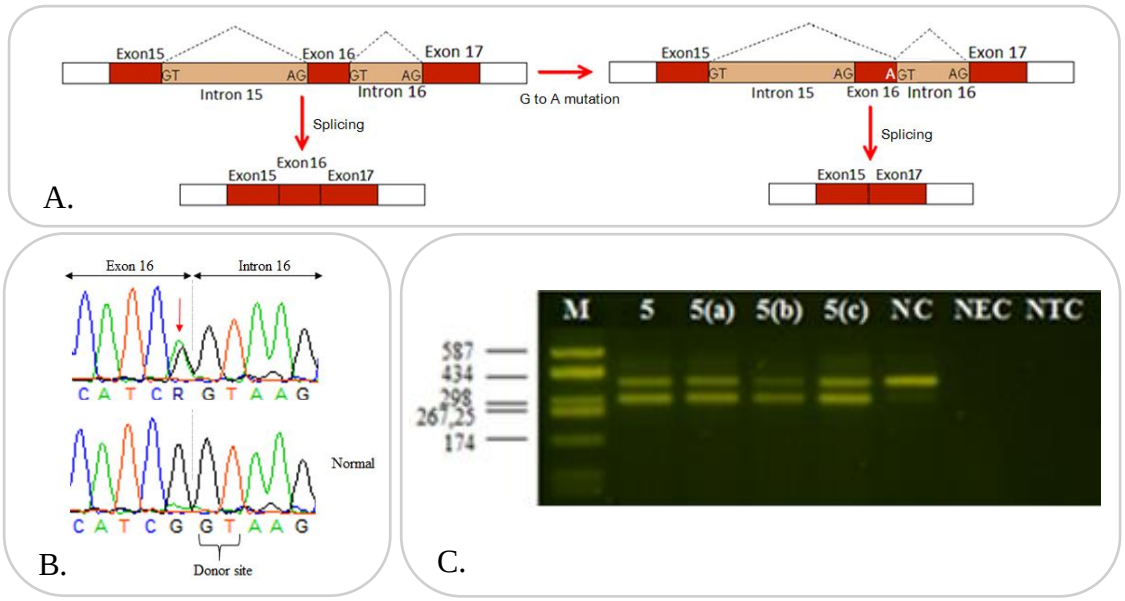

Figure 3.4 Functional study of c.2389G>A alteration. A. Schematic effect of c.2389G>A mutation in intron 16 of *LDLR* gene, resulting in exon 16 skipping. B. Comparison between DNA electropherograms of patient with c.2389G>A alteration and normalipidaemic control. C. Gel-electrophoresis of amplicons encompassing exons 15 to 18. Four samples are shown: the index case (5) and three relatives identified by cascade screening: the mother 5(a), the sister 5(b) and the grandfather 5(c). A normolipidaemic individual was used as negative control (NC). No enzyme control (NEC) and no template control (NTC) were also included in the reaction. pUC18 DNA Hae III Digest was used as ladder (M). The size (in base pairs) of each fragment is indicated on the left side.

3.1.1.2 *APOB* Gene

Only one variant was found in *APOB* gene, namely in the exon 26: p.(Arg3527Gln). This is the most common mutation found in *APOB*, which markedly reduces the affinity for the LDLR leading to increasing levels of LDL-C in circulation. The screening of relatives in this family led to the identification of one more patient carrying this mutation. ACMG classifies this variant as pathogenic.

3.1.1.3 *APOE* Gene

One variant was found in the exon 4 of *APOE* gene. p.(Arg163Cys) is located in the middle of the α-helix H4, the LDLR-binding site. Therefore, is thought to impair LDLR-binding properties of the apoE protein. [123] This variant has been previously reported in Familial Type 3 Hyperlipoproteinemia patients from other populations. [123, 124]

The distribution pattern of *APOE* polymorphisms was also investigated since it has been proven that these variations affect the affinity to lipoprotein receptors and, consequently, the clearance of dietary fat from the blood. Forty six index patients (77%) presented the most common genotype ε3/ε3. Ten patients presented the genotype ε3/ε4 (17%) and 1 the ε2/ε3 (2%). Finally, 3 have the ε4/ε4 genotype (5%).

Table 3.3 *In silico* predictions and ACMG classification for the variants found in this study

Gene	Variant		*In silico* Prediction								Overall *In Silico*	ACMG Classification
			Mutation Taster	Polyphen 2	SIFT	PROVEAN	PhyloP	HSF	FSPLICE	NNSSP		
LDLR Exon 1	c.1 A>C	p.(?)	Disease causing	Benign	Damaging	Neutral	1.985	100 (1D)	100 (1D)	100 (1D)	Non concordant	Likely Pathogenic
LDLR Exon 3	c.261G>A	p.(Trp87*)	Disease causing	NA	NA	NA	4.076	100 (3A), 100 (3D)	100 (3A), 100 (3D)	100 (3A), 100 (3D)	Not applicable	Pathogenic
LDLR Exon 4	c.551G>A	p.(Cys184Tyr)	Disease causing	Probably Damaging	Damaging	Damaging	5.438	100 (4A), 63 (4D)	100 (4A), 100 (4D)	100 (4A), 100 (4D)	Pathogenic	Pathogenic
LDLR Exon 4	c.670G>A	p.(Asp224Asn)	Disease causing	Probably Damaging	Damaging	Deleterious	5.864	96 (4A), 100 (4D)	100 (4A), 100 (4D)	100 (4A), 100 (4D)	Pathogenic	Pathogenic
LDLR Exon 4	c.693C>G	p.(Cys231Trp)	Disease causing	Probably Damaging	Damaging	Deleterious	0.329	100 (4A), 100 (4D)	100 (4A), 60 (4D)	100 (4A), 68 (4D)	Pathogenic	Likely Pathogenic
LDLR Exon 6	c.862G>A	p.(Glu288Lys)	Disease causing	Probably Damaging	Damaging	Deleterious	5.732	100 (6A), 100 (6D)	100 (6A), 100 (6D)	100 (6A), 100 (6D)	Pathogenic	Likely Pathogenic
LDLR Intron 6	c.941-2A>C	p.(?)	Disease causing	NA	NA	NA	4.761	63 (7A), 100 (7D)	0 (7A), 100 (7D)	0 (7A), 100 (7D)	Pathogenic (splicing)	Pathogenic
LDLR Intron 7	1060+1G>A	p.Gly314_Glu353del	Disease causing	NA	NA	NA	5.664	94 (7A), 69 (7D)	100 (7A), 0 (7D)	100 (7A), 0 (7D)	Pathogenic (splicing)	Pathogenic
LDLR Exon 9	c.1216C>T	p.(Arg406Trp)	Disease causing	Probably Damaging	Damaging	Deleterious	2.637	102 (9A), 100 (9D)	100 (9A), 100 (9D)	100 (9A), 100 (9D)	Pathogenic	Likely Pathogenic
LDLR Exon 9	c.1291G>A	p.(Ala431Thr)	Disease causing	Probably Damaging	Damaging	Deleterious	5.506	100 (9A), 117 (9D)	101 (9A), 100 (9D)	100 (9A), 100 (9D)	Pathogenic	Pathogenic
LDLR Exon 10	c.1374_1375del	p.(Arg458Serfs*8)	Disease causing	Benign	NA	NA	NA	100 (10A), 100 (10D)	100 (10A), 100 (10D)	94 (10A), 100 (10D)	Not applicable	Pathogenic
LDLR Exon 10	c.1432G>A	p.(Gly478Arg)	Disease causing	Probably Damaging	Damaging	Deleterious	5.429	100 (10A), 100 (10D);	101 (10A), 100 (10D)	100 (10A), 100 (10D);	Pathogenic	VUS
LDLR Exon 13	c.1886del	p.(Phe629Serfs*36)	Disease causing	Probably Damaging	NA	NA	2.151	97 (13A), 100 (13D)	100 (13A), 100 (13D)	100 (13A), 100 (13D)	Not applicable	Pathogenic
LDLR Exon 13	c.1897C>T	p.(Arg633Cys)	Disease causing	Probably Damaging	Damaging	Deleterious	1.999	100 (13A), 100 (13D)	100 (13A), 100 (13D)	100 (13A), 100 (13D)	Pathogenic	Likely Pathogenic
LDLR Exon 14	c.2054C>T	p.(Pro685Leu)	Disease causing	Probably Damaging	Damaging	Deleterious	5.719	100 (14A), 100 (14D)	100 (14A), 100 (14D)	100 (14A), 100 (14D)	Pathogenic	Pathogenic
LDLR Exon 16	c.2389G>A	p.Ala771Valfs*17	Disease causing	Benign	Tolerated	Neutral	3.306	100 (16A), 88 (16D); New 150(16A)	100 (16A), 52 (16D)	100 (16A), 89 (16D)	Non concordant	VUS
APOB Exon 26	c.10580G>A	p.(Arg3527Gln)	Disease causing	Probably Damaging	Damaging	Neutral	4.569	100 (26A), 100 (26D);	100 (26A), 100 (26D)	100 (26A), 100 (26D)	Non concordant	Pathogenic
APOE Exon 4	c.487C>T	p.(Arg163Cys)	Disease causing	Probably Damaging	Damaging	Deleterious	2.238	100 (4A)	100 (4A)	100 (4A)	Pathogenic	VUS

Table 3.4 Comparison of functional studies results for the variants found in the *LDLR* and *APOB* genes with overall in *silico* and ACMG classification.

Variants		Functional Studies			Overall *In silico*	ACMG Classification
		Assay	Product Activity	Result		
LDLR Exon 3	p.(Trp87*)	Heterologous cells (CHO), FACS assays	5-10% cell surface LDLR; 5-10% binding; 10-15% uptake [53]	Pathogenic	Not applicable	Pathogenic
LDLR Exon 4	p.(Cys184Tyr)	Heterologous cells (CHO), FACS assays	Normal cell surface LDLR;5-10% binding and uptake [125]	Pathogenic	Pathogenic	Pathogenic
LDLR Exon 4	p.(Asp224Asn)	Hmz patients' fibroblast, 125I-LDL assays	<2% LDLR activity [48]	Pathogenic	Pathogenic	Pathogenic
LDLR Exon 6	p.(Glu288Lys)	Htz patients' lymphocytes, FACS assays / Heterologous cells (CHO), FACS assays	Normal cell surface LDLR, 40-50% LDL binding and uptake / Normal cell surface LDLR, <10% binding and uptake [53/125]	Pathogenic	Pathogenic	Likely Pathogenic
LDLR Intron 7	p.Gly314_Glu353del	Htz patients' lymphocytes, RNA assays	Skipping of exon 7 [74]	Pathogenic	Pathogenic	Pathogenic
LDLR Exon 9	p.(Arg406Trp)	Heterologous cells (CHO), FACS and WB assays	60-65% LDLR cell surface, binding and internalization; reduced mature protein [54]	Pathogenic	Pathogenic	Likely Pathogenic
LDLR Exon 9	p.(Ala431Thr)	Hmz patients' fibroblast, 125I-LDL assays / COS7 cells	5-15% LDLR activity / 20% activity [101, 103]	Pathogenic	Pathogenic	Pathogenic
LDLR Exon 10	p.(Gly478Arg)	Comp htz patients' fibroblast, 125I-LDL assays	2-5% LDLR activity [48]	Pathogenic	Pathogenic	VUS
LDLR Exon 14	p.(Pro685Leu)	Hmz patients' fibroblast, 125I-LDL assays / 125I-LDL and RNA assays	15-30% /20-25% LDLR activity [112/96]	Photogenic	Photogenic	Pathogenic
LDLR Exon 16	p.Ala771Valfs*17	Htz patients' lymphocytes, RNA assays	Skipping of exon 16 [107]	Pathogenic	Non concordant	VUS
APOB Ex26	p.(Arg3527Gln)	Htz patients' LDL, U937 cells proliferation / Lymphocytes, HepG2 and U937 cells /	50 % cells proliferation / 40-50% binding and uptake and reduced LDL diameter [120/122]	Pathogenic	Non concordant	Pathogenic

3.1.2 MLPA Analysis

The assessment of large rearrangements by Multiplex Ligation-dependent Probe Amplification (MLPA) was also performed and data analysis revealed no alterations in the group of patients under study.

3.1.3 *In silico* Predictions, Functional Evidence and ACMG Classification

The results obtained by different software packages are presented in Table 3.3.

In silico predictions and functional studies results were concordant for 8 of the 11 variants with functional studies. They were not concordant for the variant c.2389G>A, p.Ala771Valfs*17 in *LDLR* with only Mutation Taster predicting its pathogenicity and for the variant c.10580G<A, p.(Arg3527Gln) in *APOB* since PROVEAN predicted the variant to be neutral. *In silico* predictions by Polyphen 2, SIFT and PROVEAN were not applicable for the intronic variants and the nonsense mutation c.261G>A, p.(Trp87*). SIFT and PROVEAN predictions were not applicable for the other two nonsense mutations.

Effects on splicing were also predicted for all variants. All missense variants were predicted to not affect splicing by these tools. Putative splicing variants were predicted to present alterations in donor or acceptor sites. *In silico* overall for splicing was also not concordant for the splicing mutation c.2389G>A, p.Ala771Valfs*17.

Nine variants (50%) presented high PhyloP values (>+4.4) revealing their rich evolutionary conservation which suggests that the aminoacids codified by these conserved nucleotides must play an important role in protein structure and function. Eight variants presented low PhyloP scores, associated with poorly preserved amino acids. For c.1374_1375del variant, PhyloP score could not be calculated, since is a deletion of two nucleotides.

All the variants were classified following ACMG recommendations. Ten of the 18 alterations were classified as pathogenic (56%). Five were classified as likely pathogenic (28%) and the remaining 3 as VUS (17%). Not all ACMG classifications were concordant with the functional studies results: only 7 were correctly classified as pathogenic (64%). Of the remaining, 2 were classified as likely pathogenic and 2 as VUS.

4 DISCUSSION

4.1 Molecular Diagnosis

Although the clinical criteria correctly identified 38% (23) of the 60 patients with familial hypercholesterolaemia, 62% fulfilled the Simon Broome criteria and didn't had a mutation in *LDLR* and/or *APOB*. Since *PCSK9* mutations are very rare, it is most likely that these patients do not have FH. These results prove the importance of the genetic study since the lipid profile is insufficient to predict who has FH.

In the paediatric group, 12 of 25 index patients (48%) had a possible pathogenic variant in one of the three studied genes. Of these 12 children, serum of 1 children was not available and, consequently, apoB and apoAI could not be characterized. The remaining 11 have an apoB/apoA1 ratio ≥0,68, correctly differentiating monogenic from polygenic dyslipidaemia in these children. In children, environmental factors as lifestyle, diet and exercise do not have the same influence as in adults where, apart from the poor diet and lack of exercise, tobacco and alcohol consumption may also interfere with the expression of the phenotype. For this reason, a relationship between lipid profile and the presence of a genetic defect is clearer, resulting in a higher genetic detection rate. In 2 of these 12 children the clinical criteria of FH could not be confirmed since no functional studies exist to prove the pathogenicity of the variants present in these patients. However, ACMG classification predicts both to be likely pathogenic.

In the adults group, a putative pathogenic variant was found in 12 of 35 index patients (34%). Only in 9 of these adults, a proven pathogenic variant was found. Two of the remaining 3 have an ACMG classification of likely pathogenic and pathogenic so the variants found are most probably the cause of the disease. The other variant was found in *APOE* and ACMG classifies it as VUS.

Cascade screening in these families allowed the identification of another 17 definite FH patients (4 children and 13 adults) and 2 possible FH patients. Cascade screening proved to be, this way, an efficient method and more cost-effective for the identification of FH patients, having identified approximately the same number of index cases yet, at a much lower cost.

4.1.2 Mutation Analysis: Identification of Variants in the *LDLR* Gene

Several possible *LDLR* functional mutations were found in 22 of the 60 index patients studied. From the 16 different possible mutations found, 10 are missense (1 with unknown effect and 9 causing an aminoacid change), 3 nonsense resulting in a premature stop codon and 3 putative splicing alterations. Patients with these variants were all found to be heterozygous. All variants will be discussed separately bellow.

4.1.2.1 Missense Mutations

c.1A>C, p.(?) exon 1

This alteration, in the signal sequence, was observed in the first nucleotide of the exon 1 corresponding to the initiation codon which could result in the absence of translation. This variant was found in one index patient and the hypercholesterolaemic mother, and has been described in other populations in patients with FH phenotype. [84-86] This variant is classified as likely pathogenic following the recommendations of ACMG. Previous studies showed that an alteration in the same aminoacid position but for a different nucleotide change (c.1A>T; p.Met1Leu) produces no detectable protein. [126] Nonetheless, only functional studies can confirm its pathogenicity meaning that the existing information is not sufficient to confirm the clinical diagnosis of FH.

c.551G>A, p.(Cys184Tyr), exon 4

This variant was found in only one index patient (age 21) without family history of CHD. The aminoacid change occurs in the ligand-binding domain and has been described in other populations in patients with FH phenotype. [74, 87-90] Functional studies performed in heterologous cells revealed that the protein presents a normal expression at the cell surface and 5-10% binding and uptake. [125] The low rate of binding and uptake of LDL confirms that the LDLR pathway is strongly affected and so, the clinical diagnosis in the index patient is confirmed. ACMG classification also classified this variant as pathogenic. The early detection of this variant in this patient can prevent the development of premature CHD.

c.670G>A, p.(Asp224Asn), exon 4

This variant was found in four apparently unrelated index patients and another four relatives were identified, being the most common variant found in this study. In Portugal, it is also one of the most common variants found. [74] Two of these four families showed a strong phenotype, with premature CHD in at least one family member. Several young FH patients were identified and premature CHD can be prevented if they start adequate therapeutic measures. The aminoacid change occurs in the domain responsible for the binding to apoB in LDL particles. This variant has been described in other patients with FH phenotype from other populations. [48] Functional studies showed that this variant in homozygous patients' fibroblasts produced a protein with less than 2% of its normal activity being considered, this way, a null allele and so, one of the most severe mutations causing FH. [48] There is no doubt that this variant is the cause of the disease. ACMG algorithm classifies this variant as pathogenic.

c.693C>G, p.(Cys231Trp), exon 4

This variant was identified in only one index patient (age 45) with very high cholesterol levels and no CHD. This variant was already described, in other populations, in patients with FH phenotype. [93-95] Although ACMG classification suggests the pathogenicity of this variant, no functional studies exist to support it. The severity of the phenotype presented by this patient and the importance of the domain affected, leads to the presumption that this mutation is the cause of the disease. However, functional studies should be performed to confirm the clinical diagnosis of FH.

c.862G>A, p.(Glu288Lys), exon 6

This variant was found in two siblings of young age (9 and 14 years old). It was already found in patients with clinical diagnosis of FH in other countries. [92, 96] Functional studies performed in heterologous cells proved that the altered protein has normal cell surface expression and <10% of binding and uptake, confirming its pathogenicity. [125] This way, the variant found confirms the clinical diagnosis of FH. ACMG classifies this variant as likely pathogenic mainly due to the insufficient information about co-segregation.

c.1216C>T, p.(Arg406Trp), exon 9

This variant was found in two index patients of unrelated families, and another two relatives. One of these families shows a strong phenotype (values of CT above 300mg/dL) with premature CHD and presence of xanthelasma in one family member. Again, several young FH patients were identified (15 and 23 years old) and development of premature cardiovascular disease can be prevented if they start adequate therapeutic measures. This variant has been described in other populations in patients with FH phenotype. [99,100] Functional studies in heterologous cells showed that the protein expression in the cell surface is reduced to 60-65% as well as the binding and internalization, proving the pathogenicity of this variant. [54] This way, clinical diagnosis of FH in these patients is confirmed by the presence of this variant. ACMG classifies this variant as likely pathogenic, which can be explained

by the co-segregation information since not all affected patients carries the mutation. However, it has been shown that patients with this alteration have a very variable phenotype. [56]

c.1291G>A, p.(Ala431Thr), exon 9

This variant was found in two index patients of two unrelated families and another relative was identified. One of these index patients showed a strong phenotype (427mg/dL of TC and 342mg/dL of LDL-C, without medication) and her family presented premature CHD in more than one family member and presence of xanthomas. This variant was already described in patients with clinical FH in other populations. [90, 95, 100-104] Functional assays performed in homozygous patients' fibroblast proved that the altered protein has a reduced activity of 5-15%. [101] More recently, transient expression in COS-7 cells revealed a reduction of the protein activity to 20%. [103] This way, the variant found confirms the clinical diagnosis of FH. ACMG classification also classifies this variant as pathogenic.

c.1432G>A, p.(Gly478Arg), exon 10

This variant was identified in one patient (age 64) with severe phenotype (457 mg/dL of TC and 376 mg/dL of LDL-C without medication). Other patients with FH phenotype in different populations present this alteration. [48, 90, 106-108] Functional studies performed in compound heterozygous (with p.(Asp342Glu)) patients' fibroblasts showed that the altered protein has 2-5% of activity in heterozygous, consistent with the aggressive phenotype of this patient. [48] Overall *in silico* predicts its pathogenicity but ACMG classifies this variant as VUS. This might be explained by the fact that functional studies in compound heterozygous are not considered in ACMG guidelines as supportive of a damaging effect on the gene or gene product, since the effect analysed might be a result of both alterations. For this reason, it is very important that this variant is assessed by functional studies, preferably by expression studies in heterologous cells.

c.1897C>T, p.(Arg633Cys), exon 13

This variant was found in one patient (age 16) and one relative. This variant was already reported in patients with FH phenotype from other countries. [109-111] Nevertheless, this is the first time that this variant is described in a Portuguese patient. ACMG classification suggest its pathogenicity. However, with no functional studies performed, the clinical diagnosis of FH cannot be confirmed for these patients.

c.2054C>T, p.(Pro685Leu), exon 14

This variant was found in only one patient (age 65) with a severe phenotype (280mg/dL of TC and 220 mg/dL of LDL-C on medication) and premature CHD (at 65 years old). FH phenotype in patients from other populations was already associated with this variant. [74, 107, 112-115] Functional studies performed in homozygous patients' fibroblasts showed that the altered protein has 15-30% of its activity, consistent with the aggressive phenotype of this patient. [112] This way, the variant found confirms the clinical diagnosis of FH. ACMG also classifies this variant as pathogenic.

4.1.2.2 Nonsense Mutations

Three nonsense mutations were found, resulting in a premature stop codon.

c.261G>A, p.(Trp87*) exon 3

This variant was found in one family. The index patient is a child (age 10) with very high levels of total cholesterol and LDL-C (394mg/dL and 332mg/dL, respectively). The cascade screening led to the identification of two relatives: the sister and the mother who already had a myocardial infraction at the age of 34. This severe phenotype can be explained by the presence of a truncated peptide due to a stop codon. Functional assays performed in heterologous cells showed that the expression of LDLR in

the cell surface is reduced to 5-10%, confirming its pathogenicity. The binding of the altered protein to the LDL particle is 5-10% and the uptake is 10-15%. [53] Furthermore, this variant was already described in other populations in patients with FH phenotype. [86] Due to the severity of the effect produced by this variant in the LDLR, the finding of this variant confirms the clinical diagnosis in the patients studied. Following ACMG recommendations, this variant is classified as pathogenic.

c.1374_1375del, p.(Arg458Serfs*8), exon 10

This variant was found in one index patient (age 42) and her two children with 23 and 10 years old. The deletion of two nucleotides (AG) results in a frameshift and in the creation of a premature codon stop at aminoacid 466. This variant was already described in patients with FH phenotype from other populations [105], but no functional studies were performed to prove its pathogenicity. However, due to the severity of the defect (premature protein termination), the clinical diagnosis was considered to be confirmed. ACMG also classifies this variant as pathogenic

c.1886del, p.(Phe629Ser fs*36), exon 13

This variant was found in one child (age 8) with severe phenotype (364mg/dL of TC and 284 mg/dL of LDL-C). Only the mother sample was available for study though the dyslipidaemia seemed to be inherited from the father. The deletion of one nucleotide (T) creates a stop codon at aminoacid 665. The clinical diagnosis was considered to be confirmed since the resultant protein is truncated. ACMG also classifies this variant as pathogenic.

4.1.2.3 Splicing Mutations

Most introns start with the sequence GT and end with the sequence AG (in the 5' to 3' direction). They are referred to as the splice donor and splice acceptor site, respectively. These two sites, together with the branch site, located 20 - 50 bases upstream of the acceptor site, enables the splicing of introns.

Mutations affecting splicing are known to be pathogenic since alteration in splicing patterns of one or multiple transcripts will disrupt the production or function of the encoded proteins. However, only the determination of the differential expression of both transcripts (normal and mutant) can lead to a better characterization of cellular defect and help to explain the difference in phenotype observed between carriers of different splicing mutations. Additionally, it can lead to the identification of new drug targets and a more precise medicine.

Three variants that might affect splicing were detected in heterozygous patients during this study.

c.941-2A>C, p.(?), intron 6

This variant was found in one patient (age 38) with a severe phenotype (408 mg/dL of TC and 332 mg/dL of LDL-C without medication) and no CHD. A single base substitution of A to C at nucleotide 941-2 causes a deletion of the acceptor site. [95] Since the splice acceptor site AG is mutated, it can be presumed that the splicing machinery will look for the next acceptor site, resulting in part or complete retention of intron 6. Nevertheless, no functional studies were performed until now and so, the clinical diagnosis of FH cannot be confirmed. ACMG classifies this variant as pathogenic.

c.1060+1G>A, p.Gly314_Glu353del, intron 7

This mutation was identified in two index patients of unrelated families and another relative was also identified. One of the index patients had a myocardial infarction at the age of 20. A single base substitution of G to A at the 5’ end of intron 7 causes a deletion of the donor site GT and, consequently, the skipping of exon 7 generating a complete different protein from exon 6 forward. The protein produced has a deletion of 40 aminoacids in the EGF precursor homology domain and a premature stop codon is generated at aminoacid 779. Functional studies were performed with heterozygous patient’s

lymphocytes and RNA assays. [74] This variant was firstly described in the Portuguese population. [98] This way, the variant found confirms the clinical diagnosis of FH. ACMG also classifies it as pathogenic.

c.2389G>A, p.Ala771Valfs*17, exon 16

This variant was found in one child (age 8) and 3 relatives. More than one family member developed premature CHD (ages 50 and 54). The single base substitution of G to A at nucleotide 2389, the last nucleotide of exon 16, creates a new acceptor site leading to skipping of exon 16 as observed during this study. The same result was achieved with previous functional studies performed in heterozygous patients' lymphocytes and RNA studies. [107] However, only Human Splicing Finder predicted this new acceptor site with a splice site score of 150% when compared to the 100% of the normal acceptor site. On the other hand, FSPLICE predicted a considerable reduction (<88%) of the donor site splice site scores from 100% to 52% for the variant site.

This mutation is likely to affect insertion of the peptide into the membrane and helix formation, thereby inhibiting anchoring of LDLR in the cell membrane. [55] Thus, the presence of this variant confirms the diagnosis of FH. Nevertheless, ACMG classifies this variant as VUS. This mutation occurs in the last aminoacid of the exon and ACMG guidelines only considers as strong evidence of pathogenicity the splice sites in the intron. For this reason, functional studies should probably be more considered in ACMG guidelines so that a more complete and correct classification can be achieved. On the other hand, *in silico* overall is not concordant. Three of four software packages predicted this variant to be benign, tolerated or neutral. This can be explained by the fact that missense variants might affect gene function through different ways: effect on the structure, folding or stability of the protein product. [57] And, as presented here, they might also cause an effect on splicing. Consequently, the possible disease-association of missense variants is difficult to predict, as we can observe for this case.

4.1.3 Mutation Analysis: Identification of Variants in the *APOB* Gene

Presently, more than 1700 *LDLR* gene mutations cause approximately 85%-90% of FH cases (http://www.ucl.ac.uk/ldlr/LOVDv.1.1.0/). However, mutations in *APOB* are thought to be responsible for FH in 5%-10% of cases in Northern European populations. [127] During this project, one variant was found in the exon 26 of *APOB* gene. One index patient and one relative were identified with c.10580G>A, p.(Arg3527Gln), the most common mutation found in *APOB*.

Assays with heterozygous patients' LDL and U937 cells showed only 50% of proliferation. [120] Since these cells do not synthesize cholesterol, requiring it in the extra cellular medium to be able to proliferate, this mutation is probably causing impairment in binding between apoB and LDLR. More recently, functional assays performed in lymphocytes and HepG2 cells, demonstrate that the binding and uptake of LDL is reduced to 50%, confirmed by proliferation assays with U937 cells. LDL size analysis by dynamic light scattering revealed a reduction in diameter of the LDL particle carrying the mutated apoB comparing to the diameter of LDL particle carrying wild type apoB, causing implications in binding affinity of apoB to the receptors. [122] The reduced affinity for apoB results in increased levels of circulating LDL-C. However, these patients have less severe LDL-C elevations compared to patients with *LDLR* gene defects, probably due to the alternative pathway for LDL clearance through apoE present in IDL cell surface that it is also a ligand for LDLR. [127]

Overall *in silico* prediction is non concordant for this alteration. However, it has been shown that these methods are poor predictors of functional variations in *APOB*. [128] Functional studies results prove its pathogenicity and ACMG also classifies it as pathogenic, so the clinical diagnosis of FH can be confirmed for these patients.

4.1.4 Mutation Analysis: Identification of Variants in the *APOE* Gene

4.1.4.1 *APOE* Mutations

IDL and VLDL return to the liver, being cleared by virtue of apoE that serves as the ligand for the LDL receptor. One variant was found in the exon 4 of *APOE* gene: c.487C>T, p.(Arg163Cys). This variant is located in the middle of the α-helix H4, the LDLR-binding site. Therefore, is thought to alter LDLR-binding properties of the apoE protein [124], causing an impairment in IDL and VLDL catabolism. This variant has been previously reported in Familial Type III Hyperlipoproteinemia patients from other populations. [124, 123] This rare combined hyperlipidaemia is characterized by high levels of cholesterol and triglycerides and a high risk of premature atherosclerosis and cardiovascular disease and can be clinical mistaken with FH. The index patient (age 58) identified with this variant was also previously diagnosed with hypertrophic cardiomyopathy (HCM), which causes thickening of the ventricle walls. However, the severe phenotype of this patient (416 mg/dL of TC and 336 mg/dL of LDL-C on statins) suggests that another genetic defect might be causing the aggressive dyslipidaemia. Concerning to family history of CHD, her father and sister died at the age of 50 and 43, respectively, with myocardial infarction.

4.1.4.2 *APOE* Genotyping

APOE polymorphisms also affect the variability of plasma lipid levels, accounting for 4% to 8% of the total variance in LDL-C levels observed in populations. [129] For that reason, it is considered to be an important factor in determining the development of atherosclerosis. E2, E3 and E4 are common protein isoforms encoded by three different alleles, ε2, ε3, and ε4. [43, 44] Carriers of the ε2 allele are less efficient in transferring VLDLs and chylomicrons from the blood plasma to the liver because of its binding properties. [42] This allele is associated with type III Hyperlipoproteinemia in homozygotes, which might explain the clinical diagnosis in patients with dyslipidaemia but without a mutation causing FH. [123, 45] None of the index patients studied was homozygous for this allele but 2% presented the genotype ε2/ε3. By contrast, carriers of the ε3 and ε4 alleles are more efficient in this process. Forty six index patients (77%) presented the most common genotype ε3/ε3. Ten patients presented the genotype ε3/ε4 (17%) and 3 the ε4/ε4 (5%).

4.2 Patients Without Mutation

After sequencing the whole coding region, the splice junctions and the promoter region of the *LDLR* gene, part of exons 26 and 29 of *APOB* and exon 4 of *APOE*, it was not possible to find a mutation in 13 individuals in the paediatric group and 23 individuals in the adults group.

4.2.1 Paediatric Group

In the paediatric group, from the 13 without a mutation only 2 had family history of CHD. This can be explained by the young age of their parents and even their grandparents. Nevertheless, in one of these families the father died from myocardial infarction (MI) at the age of 48 and in the other, the grandparent died at 58 years old, also from MI.

In this paediatric group 3 had total cholesterol above 300mg/dL without medication and 3 were under medication with total cholesterol levels below 260mg/dL. Even so, a causative mutation was not found. Although it is possible that these patients have a mutation in *PCSK9* or in other regions of *APOB* that were not studied, most likely their dyslipidaemia is of environmental cause. In fact, environmental factors as poor diet and lack of exercising can play an important role, as a high fat diet together with low physical exercising can increase plasma cholesterol levels in these children. If both children and parents have the same poor life style this would pass the false idea of an inheritance of the phenotype, since their parents will also have dyslipidaemia. However, the possibility of these children having a genetic defect in another gene involved in lipid metabolism or in cholesterol homeostasis should not be discarded.

Even if these children do not have FH they present a severe dyslipidaemia and should be followed and counselled so that their cholesterol levels are controlled, preventing serious implications as development of premature CVD.

4.2.2 Adults Group

In the adults group without a mutation, 11 had already had CHD (48%) and 12 have family history of CHD (52%). Total cholesterol levels above 300mg/dL are present in 9 individuals (39%) and 17 adults are under medication (74%). These results were not expected since all these patients had exactly the described phenotype of an FH patient. Severe phenotype with CHD at ages between 42 and 61 was observed in half of this group. One patient, medicated with statins, had actually a total cholesterol of 339 mg/dL, being resistant to medication. However, none of these individuals presented tendon xanthomas. No clinical distinction between these patients and those with a mutation in one of the three studied genes can be observed. This suggests that other factors, not only environmental but genetic defects in other genes or even unstudied regions of these genes, can be determining the phenotype.

PCSK9 is involved in the regulation of LDLR, and gain-of-function mutations in this gene will decrease the number of LDL receptors on the cell surface resulting in LDL-C accumulation in circulation. For this reason, the third phase of molecular study in the Portuguese FH study is the screening of *PCSK9* mutations in patients without a mutation in *LDLR* and *APOB* genes. The molecular study of *PCSK9* gene should be performed for patients with aggressive phenotype, although only about 1-5% of FH cases are a result of *PCSK9* gain-of-function mutations. [127]

4.3 Diagnostic Gap

As presented in this study, many clinically diagnosed FH patients fail to show any mutation in the three studied genes. The presence of mutations in other unknown and/or novel genes that are involved in cholesterol metabolism, may not be discarded. Only mapping studies, as genome-wide association studies, to discover novel genes that might be the cause of FH will fill this diagnostic gap. Another possible explanation is that the genetic defect causing FH in these patients may be present in a non-coding region of the studied genes not detected by the methodologies used in this study but that might affect the expression or RNA processing.

On the other hand, FH is a disease with a great phenotypic variability. [130] These phenotypes result from a combination of genetic, epigenetic, metabolic, and environmental factors, different for every person. Corroborating with this is the observation of people carrying the same mutation with different lipid levels. [131, 132] Finally, some patients can also be misdiagnosed having, in fact, other diseases. Polygenic hypercholesterolaemia is the most common cause of elevated serum cholesterol concentrations, with triglyceride concentrations within the reference range. This condition is caused by a susceptible genotype aggravated by one or more factors, including atherogenic diet (excessive intake of saturated fat), obesity, and sedentary lifestyle and is associated with an increased risk for CHD. Some of the patients without a mutation presented high levels of cholesterol and also triglyceride concentrations above the reference range, clinical features of familial combined hyperlipidaemia (FCHL), another common polygenic disorder.

4.4 Final considerations

The clinical diagnosis was confirmed for 19 of 60 index patients studied, giving a positive detection rate of 32%. As discussed here before, a definitive molecular diagnosis for FH is not possible for patients carrying variants without functional studies to prove their pathogenicity. This represents a serious problem for FH diagnosis and the variants identified must be functionally assessed for a definite diagnosis.

The newly identified FH patients are now receiving treatment and counselling. Monitoring these patients is important to ensure that they follow the medication correctly and to adjust the therapeutic regime if needed. This way, premature CVD, possibly leading to death, can be prevented and better quality of life can be achieved. Due to early detection of their disease through this study, and if treated correctly from early age, these children will not develop premature CHD. Thus, this study helped to determine the right therapeutic regime for each patient, improving patients' prognosis.

Nowadays, a major challenge in clinical practice is to promote medical education and awareness of FH to increase patients' identification and implementing the correct treatment of these patients. Comparing with general population, FH patients have considerably increased CVD mortality, even if treated with all available lipid-lowering drugs that should be administrated during their entire lives. The majority of these patients remain currently undetected and increasing FH awareness in hospitals and general public is important to reduce their high risk for premature CHD and death.

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Appendix I - Molecular Diagnosis of FH

Table AI.1 Proportion of reagents per mL of blood for genomic DNA extraction

Blood	TKM X100	IGEPAL	TKM1	TKM2	SDS	NaCl	EtOH
1mL	1mL	25µL	1mL	160µL	10µL	60µL	460µL

Table AI.2 PCR and Automated Sanger Sequencing – primers and annealing temperatures

Exon	5' -3' sequence	Amplified region (bp)	Annealing Temperature	Primers PCR	Primer SEQ
Promoter & LDLR Exon 1	F:ACAAATCAAGTCGCCTGCCC R:GCCATTACCCCACAAGTCTCC MB257:GGGTTAAAAAGCCGATGTCA	480	59º	SPr+1F SPr+1R	MB257
LDLR Exon 2	F:TCCCATACCCCAGAGAGTCCATA R:CAGCCGCCATCATCAAAAAG	587	58º	R2F R2R	R2F
LDLR Exon 3	F:GGTTTCACTATATTGGCCAGG R:CTCCCCAGGACTCAGATAGG	327	59º	LDL 3F1 MB260	LDL 3F1
LDLR Exon 4	F:GTACAGATGAGGAAACTGAG R:TTGGCATGTTGTTGGAAATCC R4F:GAGGAAACTGAGGCACCGAG	677	57º	EX4F EX4R	R4F
LDLR Exon 5	F:GCAAAAGGCCCTGCTTCTTT R:GAGGCTCTGAGAAGTCAAGT	342	58º	EX5F NEW EX5R NEW	EX5FNEW
LDLR Exon 6	F:TGAATGAGTGCCAAGCAAAC R:TTCCCAAAACCCTACAGCAC	277	59º	MB328 MB329	MB328
LDLR Exon 7	F:GCGAAGGGATGGGTAGGG R:GCATGAGGGGTTTGGTTG	248	58º	MB316 MB317	MB316
LDLR Exon 8	F:ATCTCCCGAGAGGCTGGGCTGTCT R:CCCGGTCAGGGGATATGAGTCTGT	361	59º	MB30 MB31	MB30
LDLR Exon 9	F:AAGGGGATGGGGAGGCACTCTTG R:CCTCATCTCACCTGCGGGCCA	397	59º	EX9+10F MB277	EX9+10F
LDLR Exon 10	F:CCTTGGCCCGCAGGTGAGA R:GTGCTGGGATTACAGGTGCTTTGA	403	62º	MB34 MB35	MB34
LDLR Exon 11	F:GCCACATTTGGAGTTTGGGGTTC R:AGCAGCTTGGGCTTGTCCCAGA	355	60º	EX11F EX11R	EX11R
LDLR Exon 12	F:GGTGCTTTTCTGCTAGGTCC R:TTTTCTGCGTTCATCTTGGCT	347	59º	EX12F EX12R	EX12F
LDLR Exon 13	F:CTAGTTGTGGAGAGAGGGTGGC R:GCGGAGTCAGGGCAGGAACGAG	275	60º	EX13F EX13R	EX13F
LDLR Exon 14	F:GAAACCTCCTTGTGGAAACTCT R:GAAAAGTATGGTTATCCCGACT	388	58º	EX14F EX14R	EX14F
LDLR Exon 15	F:CCAAGGTCATTTGAGACTTTCGT R:GAGAGAAGGTCAGCAAGGGAGTG	388	60º	EX15F EX15R	EX15R
LDLR Exon 16	F:GTCCTCTGCCTGCTCCATTTCTT R:ATCCTCCATCTGACCCCTTAGC	350	60º	EX16F EX16R	EX16F
LDLR Exon 17	F:GAGCTGGGTCTCTGGTCTCG R:GCGCACAGAAGCATTCACCT	500	60º	R17F R17R	R17F
LDLR Exon 18	F:GAGCGGTGGGAAGTGACTGAAT R:TGGTGCCATCTGCTGTTGTGTG	580	59º	EX18F EX18R	EX18F
ApoB (exon 26)	F:GAGCAGTTGACCACAAGCTTAGCTTGGAA R:GGGTGGCTTTGCTTGTATGTTCTCCGT	343	59º	P61 P62	P61
ApoB (exon 29)	F:CCAAGATGAGATCAACACAATC R:AACTTGACTTGAGAGTTGGG	334	59º	MB63 MB64	MB63
ApoE (exon4)	F: CTCTGGCTCATCCCCATCT R: ACTAGGGTCCACCCCAGGAG	938	59º	MB326 MB327	MB326

Table AI.3 PCR mix for genomic DNA amplification for one tube. (A) For *LDLR* and *APOB* genes, buffer, Mg^{2+} and TAQ enzyme from Bioline were used. Exceptionally, for the third exon of *LDLR*, the mix was prepared with equal volume of buffer and Mg^{2+} from Platinum and 0.08 µL of TAQ also from Platinum. (B) For the *APOE* gene, the GC Rich kit was used: the PCR mix is divided in master mix 1 and 2. A negative control, without DNA, was present in every PCR reaction.

(A)

Reagents	Volume
H_20	14,625 µL
dNTPs 1.25mM	4 µL
Buffer 10x	2,5 µL
Mg^{2+}	0,75 µL
Primer Fw (10pmol/µL)	1 µL
Primer Rv (10pmol/µL)	1 µL
TAQ	0,125 µL
Total	24 µL
DNA (100-200 ng)	1 µL

(B)

Reagents for Master Mix 1	Volume
dNTPs Mix 100mM	2 µL
Primer F(10pmol/µL)	1 µL
Primer Rv (10pmol/µL)	1 µL
S3 (Resolution Solution) 5M	2,5 µL
S5 (PCR Grade Water)	10,5 µL
Total	17 µL

Reagents for Master Mix 2	Volume
S2 (Reaction Buffer, 5x conc.)	5 µL
S1 (Enzyme Mix, 2U/µL)	0,5 µL
S5 (PCR Grade Water)	2 µL
Total	7,5 µL

Table AI.4 PCR for cDNA analysis – primers and annealing temperatures

Exon	5'- 3' sequence	Amplified region (bp)	Annealing Temperature	Primers PCR	Primer SEQ
Exon 5 – Exon 9	F: CCAGTGCTCTGATGGAAACTGC R: GCCGGTTGGTGAAGAAGAGGTA	318	62º	MB25 MB13	MB25
Exon 15-Exon 18	F: GGGGCCACCCCTGGGCTCAC R: AAGGCCGGCGAGGTCTCAGGA	381	62º	MB20 MB21	MB20

Table AI.5 PCR mix for cDNA amplification for one tube.

Reagents	Volume
H_2O DEPC	14,67µL
dNTPs	4µL
Buffer Platinum	2,5µL
Mg^{2+} Platinum	0,75µL
Primer Fw a 10ρmol/µL	1µL
Primer Rv a 10ρmol/µL	1µL
TAQ Platinum	0,08µL
Total	24µL
cDNA	1,5µL

Appendix II - Clinical Diagnosis

Table A II.1 Clinical and biochemical characterization of studied index cases.

Patient	Age	Sex	Biochemical Values								CV events	Family history of CHD	Family history of high cholesterol	Medication
			TC (mg/dL)	**LDL-C** (mg/dL)	**HDL-C** (mg/dL)	**TG** (mg/dL)	**Lp(a)** (mg/dL)	**ApoA1** (mg/dL)	**ApoB** (mg/dL)	**ApoA1/ ApoB**				
1	3	F	269	207	48	82	8,3	139,0	160,0	1,15	No	No	Yes	No
2	5	F	235	171	41	145	129,00	129,50	135,38	1,05	No	No	Yes	No
3	7	M	272	185	82	76	12,6	186,0	120,0	0,65	No	No	Yes	Omega-3
4	8	M	311	224	85	71	242,1	198,0	162,0	0,82	No	No	Yes	No
5	8	F	289	232	51	52	24,3	128,0	162,0	1,27	No	Yes	Yes	No
6	8	F	282	217	55	121	10,6	151,0	152,0	1,01	No	No	Yes	Omega-3
7	8	M	230	170	50	82	78,1	127,0	122,0	0,96	No	No	Yes	N
9	8	F	216	170	38	56	10,4	98,2	122,2	1,24	No	Yes	Yes	Stanols + Phytosterol
8	9	F	309	224	49	241	8,3	174,0	153,0	0,88	No	No	Yes	No
10	9	M	188	131	38	126	34,6	111,0	106,0	0,95	No	No	Yes	No
11	10	F	262	189	67	42	8,3	139,0	141,0	1,01	No	No	Yes	No
12	10	F	217	164	31	96	40,8	91,0	139,0	1,53	No	Yes	Yes	Statins
13	10	F	251	183	49	66	135	129,4	136,9	1,06	No	No	Yes	Statins
14	10	M	270	205	58	61	Serum not available				No	Yes	Yes	Stanols + Phytosterol
15	11	M	286	187	83	78	Serum not available				No	No	Yes	Stanols + Phytosterol
16	11	M	214	143	55	139	174,9	140,7	122,4	0,87	No	No	Yes	Statins
17	12	M	190	120	53	66	120,7	127,0	96,0	0,76	No	Yes	Yes	No
18	12	M	257	174	68	32	< 8,3	151,0	119,0	0,79	No	No	Yes	Statins
19	13	M	190	134	51	35	< 8,3	127,0	89,0	0,70	No	No	Yes	Statins
20	14	F	222	149	54	101	19,8	134,0	117,0	0,87	No	No	Yes	No
21	15	F	275	189	77	56	13,6	159,0	137,0	0,86	No	No	Yes	No
22	15	F	272	187	83	96	60,0	185,0	137,0	0,74	No	No	Yes	Omega-3
23	15	F	182	96	66	134	90,3	193,0	97,0	0,50	No	No	Yes	Statins
24	15	F	274	199	64	111	59,6	153,0	136,0	0,89	No	Yes	Yes	Statins
25	16	F	196	129	47	75	14,4	110,0	106,5	0,97	No	No	Yes	No

Patient	Age	Sex	Biochemical Values								CV events	Family history of CHD	Family history of high cholesterol	Medication
			TC (mg/dL)	**LDL-C** (mg/dL)	**HDL-C** (mg/dL)	**TG** (mg/dL)	**Lp(a)** (mg/dL)	**ApoA1** (mg/dL)	**ApoB** (mg/dL)	**ApoA1/ ApoB**				
26	20	F	172	100	56	87	0,80	127,2	81,3	0,64	MI	No	Yes	Statins
27	21	F	286	198	61	161	< 8,3	206,0	159,0	0,77	No	Yes	Yes	No
28	21	M	314	226	67	88	H	157,9	146,2	0,93	No	No	Yes	Stanols + Phytosterol
29	23	F	265	175	59	193	21,6	195,0	145,0	0,74	No	Yes	Yes	Statins
30	29	M	184	123	43	114	18,7	131	93	0,71	No	?	?	Statins
31	36	M	231	155	43	159	128,9	123,0	147,0	1,20	MI, PTCA (36Y)	Yes	Yes	Statins
32	36	M	339	245	58	183	99,2	179,0	188,0	1,05	No	?	?	Statins
33	39	F	274	173	88	135	66,8	221,0	154,0	0,70	No	?	?	Ezetimebe
34	41	F	293	211	77	56	72,4	180,0	168,0	0,93	No	No	Yes	No
35	41	M	300	215	70	145	55,8	163,0	155,0	0,95	MI, PTCA, Angina	?	?	No
36	41	M	256	177	50	123	4,8	140,3	132,8	0,95	No	No	Yes	Statins
37	42	F	416	321	52	160	8,3	140,0	238,0	1,70	No	No	Yes	Statins+ Ezetimibe
38	44	M	183	108	62	98	11,4	151	85	0,6	No	Yes	Yes	Statins
39	45	F	438	357	55	119	65,1	141,0	250,0	1,77	Angina (45Y)	No	Yes	LDL apheresis
40	45	M	256	202	27	130	30,8	88,9	148,7	1,67	MI (42Y)	Yes	Yes	Statins
41	46	M	234	179	31	202	1,4	110,4	161,8	1,47	MI (43Y)	Yes	Yes	Statins
42	47	M	285	191	48	311	< 8,3	160,0	163,0	1,02	No	No	Yes	Ezetimebe
43	50	M	227	146	62	120	61,9	155,6	122,3	0,79	MI (45Y)	Yes	Yes	Statins + Phytosterol
44	51	F	257	156	57	247	252,8	168,0	157,0	0,93	MI, PTCA (48Y), Angina, TIA	No	Yes	Statins+ Ezetimibe + LDL apheresis
45	51	M	164	71	57	177	73,2	139,2	94,5	0,68	MI (48Y)	Yes	Yes	Statins+ Ezetimibe
46	52	M	260	178	40	304	-	-	-	-	MI (46Y)	Yes	Yes	Statins
47	53	F	278	195	48	150	12,5	158,0	141,0	0,89	No	Yes	?	No
48	54	F	255	173	63	82	17,4	157,0	131,0	0,83	No	No	Yes	Statins
49	55	F	165	92	54	127	54,9	155,0	88,0	0,57	CVA (46Y), MI, PTCA (54Y)	Yes	Yes	Statins
50	56	F	271	199	61	145	84,5	163,0	139,0	0,85	No	Yes	Yes	Statins
51	57	M	234	146	52	169	34,3	161,0	115,0	0,71	No	No	Yes	Statins
52	58	F	194	112	64	104	8,3	148,0	98,0	0,66	No	?	?	?
53	58	F	416	338	65	137	70,7	168,6	181,3	1,08	HC (40Y)	Yes	Yes	Statins
54	60	M	184	129	36	96	< 8,3	140,0	106,0	0,76	MI (55Y)	Yes	Yes	Statins

55	60	F	276	185	79	75	103,4	204,0	148,0	0,73	No	Yes	?	Statins+ Ezetimibe
56	62	M	167	103	47	126	39,2	135,6	96,3	0,71	MI (61Y)	?	?	Statins
57	63	F	228	159	49	120	18,1	139,0	154,0	1,11	Angina (61Y)	Yes	?	Statins+ Ezetimibe
58	64	F	457	376	32	135	97,3	106,0	276,0	2,60	No	No	Yes	No
59	65	F	280	220	46	83	24,5	127,0	163,0	1,28	Angina, CABG (65Y)	No	?	Statins+ Ezetimibe
60	66	M	190	104	59	99	57,7	192,0	94,0	0,49	TIA (57Y)	No	Yes	Stanols + Phytosterol

TC, total cholesterol; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, triglyceride; Lp(a), Liproprotein (a); ApoAI, Apolipoprotein AI; ApoB, Apolipoprotein B; MI, Myocardial infarction; CABG, Coronary artery bypass grafting; PTCA, Percutaneous transluminal coronary angioplasty, TIA, Transient ischemic attack; CVA, Cerebrovascular accident; HC, Hypertrophic cardiomyopathy. Note: Biochemical values under cholesterol-lowering medication are presented for medicated patients.